Green algae in soil: assessing their biodiversity and biogeography with molecular-phylogenetic methods based on cultures

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Contents

General Introduction

Importance of soil microalgae

Terrestrial eukaryotic microalgae were recognized as a part of soil microflora in the late 19th century and were introduced into soil ecology during the second half of the 20th century (Gollerbach and Shtina 1969; Shtina and Gollerbach 1976; Metting 1981; Starks *et al.* 1981). As photoautotrophs, microalgae constitute the basis of soil food webs and play a key role in soil development (Zenova and Shtina 1990; Zenova *et al.* 1995; Hu *et al.* 2002; Hu *et al.* 2003b; Rahmonov and Piątek 2007; Langhans *et al.* 2009; Viles 2012). The role of terrestrial eukaryotic microalgae in geomorphic processes was discussed by Viles (2012). Changes in algal abundance and diversity enable biological monitoring of soil recovery after anthropogenic pollution and contamination (Megharaj *et al.* 1986; Dahlin *et al.* 1997; Megharaj *et al.* 2000; Bérard *et al.* 2004; Krivorotov and Bukareva 2005; Nagy *et al.* 2005; Novakovskaya and Patova 2007; Kalinowska and Pawlik-Skowrońska 2010; Temraleeva *et al.* 2011; Hastings *et al.* 2014), or during postpyrogene soil development (Scherbina *et al.* 2014). Some authors recognized the importance of algal diversity in quality assessment of agricultural ecosystems (Shields and Durrell 1964; Gollerbach and Shtina 1969; Kabirov 1993; Hastings *et al.* 2014) and of land usage (King and Ward 1977). Particular species of soil microalgae are considered to be biological indicators (Balezina 1974; Shtina 1990; Megharaj *et al.* 2000; Bérard *et al.* 2005; Krivorotov and Bukareva 2005; Zancan *et al.* 2006; Dotsenko 2008), classified as a part of ecologically meaningful algocenoses (Gollerbach and Shtina 1969; Zenova *et al.* 1995; Kuzyakhmetov 1998; Şalaru *et al.* 2008; Kabirov *et al.* 2010). The interest in soil microalgae resulted in a series of comprehensive reviews on algal taxonomy, diversity, ecology and geographic distribution (Reisigl 1964; Gollerbach and Shtina 1969; Shtina and Gollerbach 1976; Metting 1981; Starks *et al.* 1981; Shtina 1990; Ettl and Gärtner 1995; Ettl and Gärtner 2014).

Distribution of soil microalgae: a microscopy-based approach

Our knowledge of algal species diversity in soils is still based mostly upon morphological observations accomplished via light microscopy of mixed or monoclonal algal cultures. Since the major fraction of algal species is invisible by direct microscopy of soil particles, algal growth should be first triggered via enrichment culturing techniques (Starks *et al.* 1981; Ettl and Gärtner 1995). The growth of particular species of microalgae is differentially favored according to the chemical composition of culturing media, or whether algae are cultured in liquid or agarized

media. The most widely used medium for culturing of terrestrial microalgae, the Bold´s Basal Medium (BBM; Bischoff and Bold 1963), is selective in favor of easy-growing (generalist) rather than rare species (Broady 1996). Our picture of the algal diversity in soils is incomplete, not only due to selective culturing techniques but also because of the demanding morphological determination of similarly looking species.

Research on diversity of edaphic microalgae has a long tradition particularly in Europe, beginning in the 19th century (Kützing 1843; Hansgirg 1888). In fact, most of the early recognized microscopic algae are also known from soils, e.g., *Stichococcus* (Nägeli 1849), *Chlorella* (Beijerinck 1893), *Hormidium*/*Klebsormidum* (Kützing 1843), *Chlamydomonas* (Ehrenberg 1833), *Coccomyxa* (Schmidle 1901), *Scenedesmus* (Meyen 1829) or *Pleurastrum*/*Leptosira* (Borzì 1895). Apart from several studies from British Isles (Bristol 1920; 1927; Lund 1945; Lund 1947), the most biodiversity research on soil microalgae was conducted in continental Europe. Considering terrestrial ecosystems of Central and Western Europe, species lists of edaphic microalgae were revealed from forests (Trenkwalder 1975; Komáromy 1983; Kostikov *et al.* 2001a; Hoffmann *et al.* 2007; Škaloud 2009), grasslands (Komaromy 1976; Komáromy 1983; Neustupa 2001), agricultural fields (Lukešová 1993; Zancan *et al.* 2006), urban regions (Perútková 2014), contaminated (e.g., post-mining) areas (Lukešová and Komárek 1987; Lukešová and Hoffmann 1996; Frouz *et al.* 2001; Lukešová 2001; Neustupa and Škaloud 2004; Kalinowska *et al.* 2008; Trzcińska and Pawlik-Skowrońska 2008), subalpine soils (Rosa and Lhotský 1971; Lukešová *et al.* 2010), alpine soils (Reisigl 1958; 1964; Türk and Gärtner 2001; Peer *et al.* 2010) and xeric sand soils (Hoppert *et al.* 2004; Rahmonov and Piątek 2007; Langhans *et al.* 2009). Particular attention was paid to the edaphic microalgae in Eastern Europe and Russia, focusing on characteristic habitats including steppe grasslands (Musabaeva 2009), steppe forests (Kostikov *et al.* 2001b; Maltseva 2005b; 2005a; 2007; Maltsev 2013; Scherbina *et al.* 2014), Carpathian and other forests (Chornevych *et al.* 2008; Iljushenko 2008; Posrednikova *et al.* 2009; Nikorych and Chornevych 2012; Maltsev and Negrulja 2013), Russian tundra (Novakovskaya and Patova 2008; Patova and Dorokhova 2008), agricultural ecosystems (Asfandijarova 2008; Şalaru *et al.* 2008; Maltseva *et al.* 2009), disturbed soils (e.g., post-mining) (Novakovskaya and Patova 2007; Bachura and Khramchenkova 2008), urban areas (Soare and Dobrescu 2010; Shekhovtseva 2014; Dorokhova *et al.* 2015), or islands in the Black Sea (Vinogradova and Darienko 2008; Darienko 2012).

The best sampled region outside Europe is North America, where the investigators focused on algal communities of mountainous regions (Bischoff and Bold 1963; Khaybullina *et al.* 2010), deserts (Flechtner *et al.* 1998; Flechtner *et al.* 2005; Flechtner *et al.* 2008), xeric sandy soils

(Smith *et al.* 2004), contaminated soils (Maxwell 1991; Nagy *et al.* 2005), serpentine soils (Terlizzi and Karlander 1979) and soils of agricultural landscapes (Fairchild and Willson 1967; Hunt *et al.* 1979; Metting and Rayburn 1979; Metting 1981). Microalgae of desert soil crusts were further studied in Africa (Büdel *et al.* 2009; Mansour and Shaaban 2010) and Asia (Islam 1960; Friedmann *et al.* 1967; Novichkova-Ivanova 1972; Chunxiang and Yongding 2003; Hu *et al.* 2003b; Al-Fredan and Fathi 2007; Bhatnagar *et al.* 2008). A lot of attention was paid to the edaphic microflora of the extremely cold and dry polar regions including both the Arctic (Androsova 1964; Broady 1978; Elster *et al.* 1999; Kaštovská *et al.* 2005; Stibal *et al.* 2006; Kaštovská *et al.* 2007; Matula *et al.* 2007; Kim *et al.* 2008; Patova and Dorokhova 2008) and the Antarctic (Broady 1979a; Broady 1984; Broady 1989; 1996; Mataloni *et al.* 2000; Cavacini 2001; Fermani *et al.* 2007). The least known is the algal diversity in humid tropical soils and so far we are aware of investigations from South and Central America (Archibald 1972; Archibald and Bold 1975; Tell 1976; Büdel 2003; Spitzer *et al.* 2014), Asia (MacEntee *et al.* 1977; Watanabe 1983; Reynaud and Lumpkin 1988; Ray and Thomas 2012; Lin *et al.* 2013), Australia and Oceania (Arvik and Willson 1974; Carson and Brown 1976; MacEntee *et al.* 1977; Carson and Brown 1978; Watanabe 1983). The edaphic microflora of small and isolated islands is almost unknown, except for e.g. St. Paul's Rocks in the equatorial Atlantic Ocean (Smith *et al.* 1974).

Phylogenetic diversity of soil microalgae

Considering the modern system of Eukaryotes, soil microalgae comprise photoautotrophic microorganisms that are phylogenetically nested within five kingdoms (Archeplastida, Excavata, Stramenopila, Alveolata and Cryptophyceae; **Fig. 1**). Archaeplastida (Adl *et al.* 2005)—mainly Chlorophyta and Streptophyta (Friedl and Rybalka 2012; Leliaert *et al.* 2012)—represents a dominant group of the most soil types (Broady 1979b; Metting 1981; Hoffmann 1989; Megharaj *et al.* 2000; Lukešová 2001; Zancan *et al.* 2006; Trzcińska and Pawlik-Skowrońska 2008; Freeman *et al.* 2009; Lin and Wu 2014). Stramenopila (Patterson 1989) are as well abundant in soils, represented by the classes Bacillariophyceae (Lukešová and Komárek 1987; Fermani *et al.* 2007; Darienko 2012; Stanek-Tarkowska and Noga 2012), Xanthophyceae (Broady 1979b; Maltseva 2005b; 2005a; Posrednikova *et al.* 2009; Novis *et al.* 2015) and Eustigmatophycae (Neustupa 2001; Khaybullina *et al.* 2010; Darienko 2012; Scherbina *et al.* 2014). Three further groups were sporadically recorded from soils: Excavata-Euglenophyta (Ashley *et al.* 1985; Maltseva 2005a; Şalaru *et al.* 2008; Lukešová *et al.* 2010; Lang *et al.* 2011), Alveolata-Dinophyta (Ettl and Gärtner 1995; Kutovaya *et al.* 2012) and Cryptophyceae-incertae sedis (Paulsen *et al.* 1992; Ettl and Gärtner 1995). Across the temperate zone, particularly the Green algae

(Chlorophyta lineage) are highly abundant in soils (Sumbali and Mehrotra 2009), whereas in tropics Cyanobacteria dominate (Roger and Reynaud 1982; Lin *et al.* 2013). It is widely accepted that variation of key physico-chemical parameters in soils (pH, organic carbon, nitrogen) affect green algae less than other microalgae (Metting 1981; Starks *et al.* 1981; Hoffmann 1989). Examples from temperate forests and grasslands show, that alkaline and nutrient rich soils favor higher group diversity comprising Cyanobacteria, Diatoms, Xanthophytes and Green algae, whereas rather acidic and nutrient poor soils are often inhabited only by Green algae (Sumbali and Mehrotra 2009; Nikorych and Chornevych 2012). Compared to different terrestrial microhabitats (hard natural or artificial substrates, tree bark, deadwood), the diversity of microalgae in soils is much higher and rarely dominated by a single group (Hallmann *et al.* 2011b; Kulichová *et al.* 2014). In soils of temperate regions, usually several groups of microalgae co-dominate.

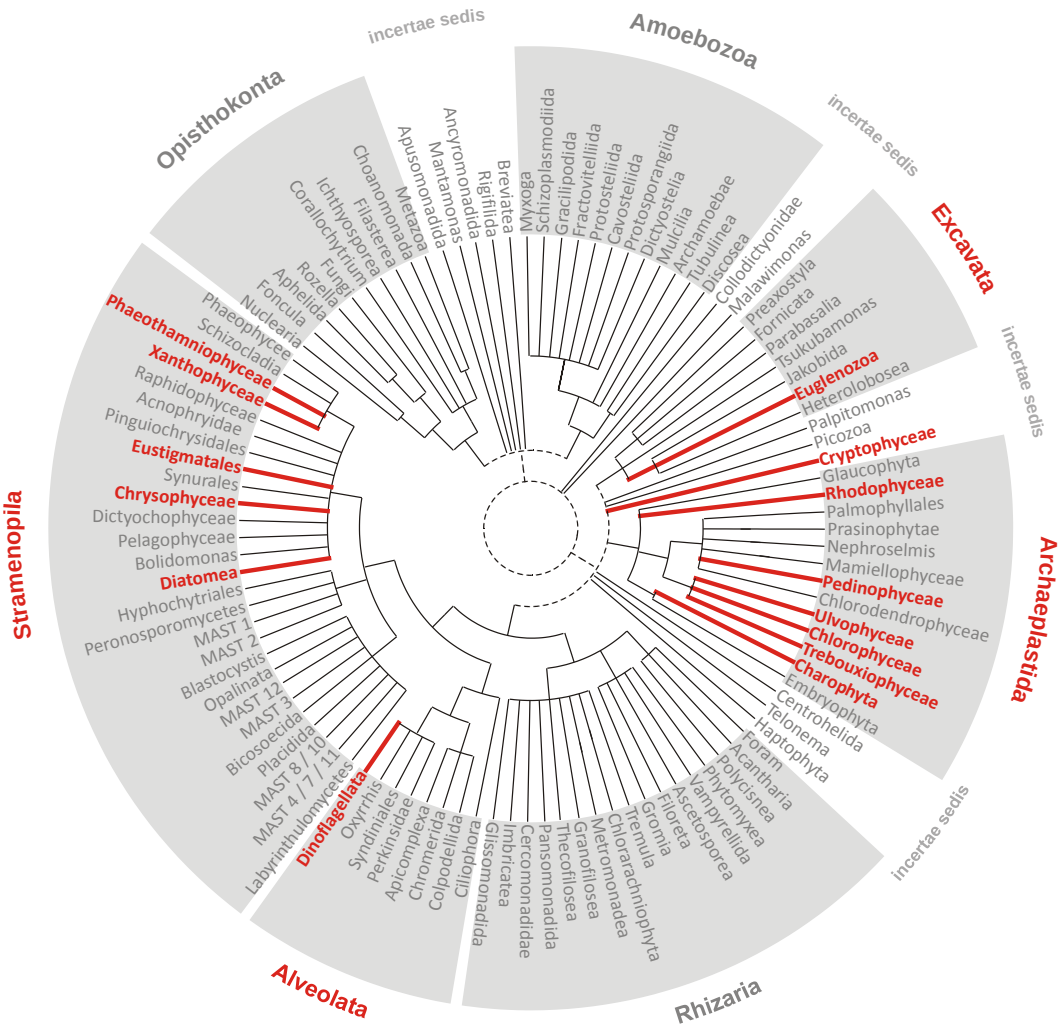

Figure 1. Schematic tree of Eukaryotes and their major lineages. Red branches represent algal lineages which include species living in soils. Adopted and modified after del Campo *et al.* (2014).

Molecular diversity of soil Green microalgae

Considering modern systematics of Green algae, we recognize 20 lineages, which comprise terrestrial species (Rindi *et al.* 2009; Friedl and Rybalka 2012; Leliaert *et al.* 2012), **Fig. 2**. The most terrestrial green microalgae belong to the Chlorophyta lineage, which has an ancestor in common with the Streptophyta (the estimated split of both lineages is in Neoproterozoic; Becker 2013). Whereas the Streptophyta (= primarily freshwater lineage) colonized terrestrial habitats at first, Chlorophyta (= primarily marine lineage) colonized land later on (Becker and Marin 2009). The molecular evidence of green algal diversity in terrestrial habitats is still scant. The most molecular data were so far retrieved from soils, e.g., Alpine glacier forefields (Frey *et al.* 2013), Siberian permafrost (Vishnivetskaya 2009), Himalaya (Schmidt *et al.* 2011; Schmidt and Darcy 2014), North-American desert crusts (Lewis and Flechtner 2002; Lewis and Lewis 2005; Flechtner *et al.* 2013) and mountains (Freeman *et al.* 2009), Atacama desert volcano (Costello *et al.* 2009), Ecuadorean rainforest (Faßhauer *et al.* 2011; Spitzer *et al.* 2014) and Antarctica (Lawley *et al.* 2004; Schmidt *et al.* 2011). A few molecular diversity studies recovered green microalgae in different aero-terrestrial habitats, e.g., European tree barks (Kulichová *et al.* 2014; Hallmann *et al.* in prep.-a) and epilithic biofilms (Horath and Bachofen 2009; Hallmann *et al.* 2011b; Ragon *et al.* 2012; Hallmann *et al.* 2013a), Ecuadorean rainforest tree barks and tree leaves (Faßhauer *et al.* 2011; Spitzer *et al.* 2014) or South and Central American sloth hairs (Suutari *et al.* 2010). Central European soils were intensively studied by microscopy-based approach, however, no molecular diversity studies were published so far. Consequently, molecular taxonomy studies on soil Green algae are scant as well. A few genera of Green

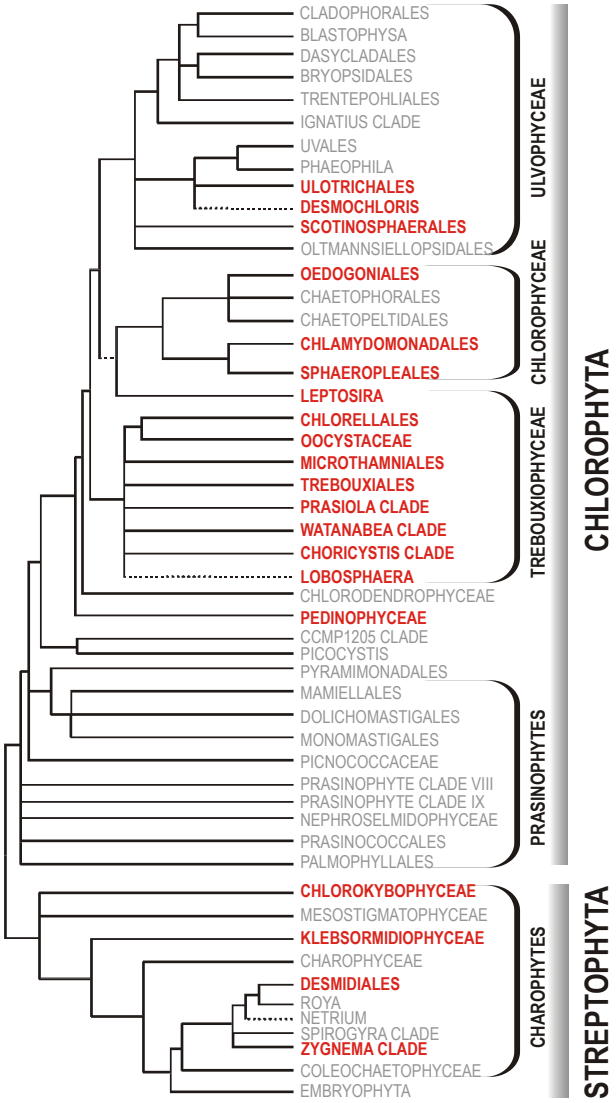

Figure 2. Schematic tree of Chlorophyta, Streptophyta and their major lineages. Red letters represent algal lineages which include soil dwelling species. Adopted and modified after Leliaert *et al.* (2012).

algae, which underwent taxonomic revisions, comprise species confirmed in European soils, e.g., *Bracteacoccus* (Fučíková *et al.* 2012), *Chlorella* (Luo *et al.* 2010; Bock *et al.* 2011; Treves *et al.* 2013), *Coccomyxa* (Darienko *et al.* 2015), *Chloropyrula* (Gaysina *et al.* 2013), *Chlamydomonas* (Pröschold *et al.* 2001), *Chloroidium* (Darienko *et al.* 2010), *Chromochloris* (Fučíková and Lewis 2012), *Dictyococcus* (Fučíková *et al.* 2011a), *Interfilum* (Mikhailyuk *et al.* 2008), *Klebsormidium* (Škaloud *et al.* 2014a), *Neocystis* (Eliáš *et al.* 2013), *Pseudomuriella* (Fučíková *et al.* 2011a; Fučíková *et al.* 2011b) and *Scenedesmus*-relatives (Lewis and Flechtner 2004; Hegewald *et al.* 2013; Kaufnerová and Eliáš 2013). Some terrestrial microalgae were described elsewhere and re-detected in European soils, but are still in need of taxonomic revisions, e.g., *Stichococcus* (Neustupa *et al.* 2007; Hodač *et al.* subm.), *Jenufa* (Němcová *et al.* 2011; Hodač *et al.* 2012), *Xylochloris* (Neustupa *et al.* 2011; Hodač *et al.* 2012). Unique Green microalgae were so far recorded from North American desert soils, e.g., *Bracteamorpha, Rotundella*, *Tumidella* (Fučíková *et al.* 2013), *Desertella*, *Eremochloris*, *Xerochlorella* (Fučíková *et al.* 2014), *Flechtneria* (Sciuto *et al.* 2015) and *Tetraflagellochloris* (Barsanti *et al.* 2013). Green algal species descriptions from African or polar soils are still extremely rare and include *Desmochloris* from Namib Desert (Darienko *et al.* 2009) or *Pabia* from the Antarctic (Friedl and O'Kelly 2002).

Biogeography of soil Green microalgae

In Green microalgae, unambiguous recognition of the most species is not yet possible, due to a lack of morphological features and because molecular taxonomy do not follow any consensual species concept. Microalgae in terrestrial habitats exhibit morphological convergence (López-Bautista *et al.* 2007), i.e., a tendency to evolve simple globular or filamentous forms throughout unrelated phylogenetic groups (**Fig. 3**). Demanding morphological determination of similarly looking species lead to general underestimation of Green algal diversity and to putative misinterpretations of their distribution patterns (Hoffmann 1989; Rindi *et al.* 2009; Sharma and Rai 2010; Rindi *et al.* 2011; Guiry 2012; Škaloud *et al.* 2015). Whereas in aquatic protists biogeography is widely accepted (Finlay and Clarke 1999; Šlapeta *et al.* 2006; Řezáčová and Neustupa 2007; De Wever *et al.* 2009; Naselli-Flores and Padisák 2015), biogeography of terrestrial Green microalgae is still far from well explored (Lawley *et al.* 2004; Sharma *et al.* 2007; Rindi *et al.* 2009; Leliaert *et al.* 2012; Bates *et al.* 2013; Ryšánek *et al.* 2014). Terrestrial Green microalgae can be encountered nearly everywhere around the globe. Particularly soil species are capable of survival after long periods of desiccation (Trainor 1970; Buzer *et al.* 1985; Trainor 1985; Trainor and Gladych 1995) and can photosynthesize even in cave darkness (Kol 1967). Their resting cysts may easily survive transportation over long distances in the air being

resistant to drought, high as well as low light intensities and high UV radiation, e.g., due to the presence of thickened cell walls or light protection pigments (Řezanka *et al.* 2004; Karsten *et al.* 2005; Häubner *et al.* 2006; Gustavs *et al.* 2010). Early morphology-based attempts comparing geographic distributions of soil microalgae, e.g. Feher (1948), as well as later studies concordantly suggested that a couple of soil algae might be cosmopolitan (Metting 1981; Starks *et al.* 1981; Hoffmann 1989), **Fig. 3**. The putative cosmopolitanism of some microalgae was in accordance with the *neutral dispersal model* (Finlay and Clarke 1999; Finlay 2002; Fenchel and Finlay 2004), which claimed that protists generally do not have biogeography. This view changed rapidly with the era of molecular phylogenies (Sharma and Rai 2010). Molecular markers enabled description of a plethora of morphologically indistinguishable species (Boenigk *et al.* 2005; Rindi *et al.* 2008; Dal Grande *et al.* 2014). As a result, microalgal biogeography seemed to be supported by cryptic species mainly observed within a restricted area. The biogeographic evidence comprises multiple algal lineages such as (1) Stramenopila, e.g., *Spumella* (Boenigk *et al.* 2005), *Xanthonema* (Rybalka *et al.* 2009; Rybalka *et al.* 2013), *Synura* (Škaloud *et al.* 2014b), *Frustulia* (Veselá *et al.* 2012); (2) Streptophyta, e.g., *Micrasterias* (Jurdíková *et al.* 2014), *Klebsormidium*, (Ryšánek *et al.* 2014; Škaloud *et al.* 2014a) and (3) Chlorophyta, e.g., *Asterochloris* (Řídká *et al.* 2014; Škaloud *et al.* 2015). However, more extensive samplings of molecular data from geographically remote regions already confirmed that at least some species of terrestrial Green microalgae are cosmopolitan. A wide geographic distribution might apply for free living species of *Coccomyxa* (Darienko *et al.* 2015) and *Klebsormidium* (Ryšánek *et al.* 2014), as well as for lichenized species of *Dictyochloropsis* (Dal Grande *et al.* 2014) and *Diplosphaera* (Fontaine *et al.* 2013). In conclusion, biogeography studies on Green microalgae still have to cope with immense lack of molecular diversity data, since only

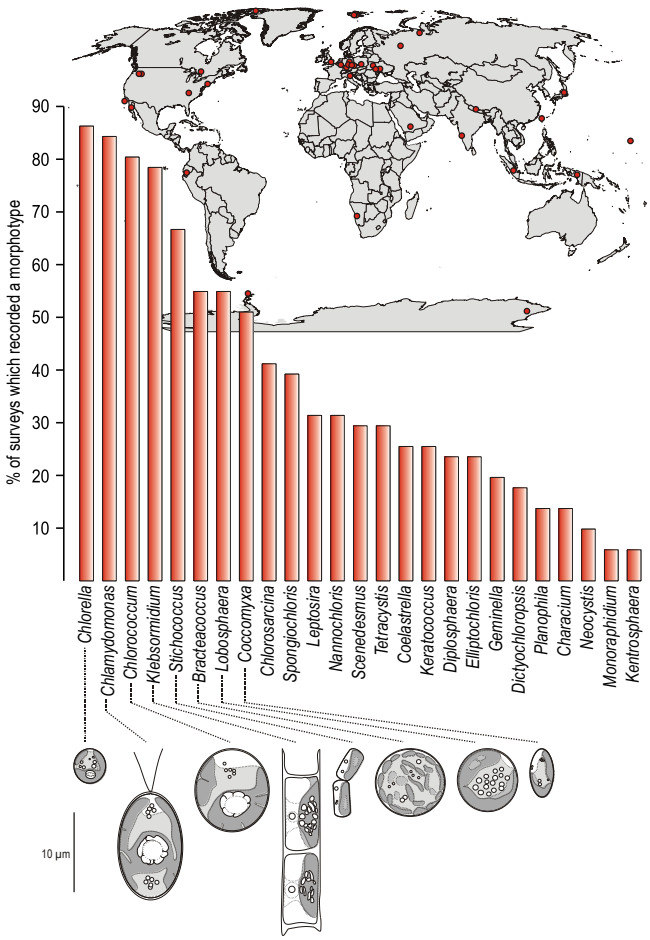

Figure 3. Bar chart summary of the most frequently recorded Green algal morphospecies/morphotypes. Species occurrences were derived from 46 floristic studies (shown in the world map and mentioned on pages 2-3).

a few regions have been sampled so far (Novis *et al.* 2008; De Wever *et al.* 2009; Kulichová *et al.* 2014). Species richness of eukaryotic microorganisms is generally poorly understood and diversities revealed from environmental samples significantly differ when using culture-dependent or culture-independent approaches (Moon-van der Staay *et al.* 2006; Doherty *et al.* 2007; Lara *et al.* 2007; Hallmann *et al.* 2009; Hallmann *et al.* 2013a; Spitzer 2013). Particularly in Green microalgae, the link between culture-independent and culture-dependent diversities has been rarely studied. To establish a link between the both approaches, more monoclonal cultures/strains need to be sequenced and phylogenetically characterized. Although there is no universal barcoding marker for identification of microalgae, plastid and mitochondrial markers are widely used, along with the nuclear SSU (e.g. V4/V9 hypervariable regions) and the internal transcribed spacers ITS1-5.8S-ITS2 (Hall *et al.* 2010; Bock *et al.* 2011; Buchheim *et al.* 2011; Fučíková *et al.* 2011b; Krienitz and Bock 2012; Wolf *et al.* 2013; Darienko *et al.* 2015; Darienko and Pröschold 2015; Heeg and Wolf 2015; Lutz *et al.* 2015; Sciuto *et al.* 2015).

General aims

(1) Biodiversity of terrestrial Green microalgae | Chapters 1-2

Traditional morphospecies-based studies revealed a considerable diversity of Green microalgae in terrestrial habitats of Central Europe. Terrestrial Green algae in temperate climate zones, however, are still poorly explored using molecular techniques, in contrast to those from less favorable environments, e.g. hot desert soils. We expect molecular methods to reveal high algal diversities in moist soils of temperate grasslands and forests as well as in submerged algal biofilms. By focusing on cultures of Green microalgae from Central European soils and semi-terrestrial biofilms, we aim to investigate genetic diversity of microscopically hardly distinguishable morphospecies.

(2) Biogeography of terrestrial Green microalgae | Chapters 3-4

Terrestrial Green microalgae are able of long-distance dispersal by resisting harsh environmental stresses. Such 'airborne' microalgae might dwell in soils and terrestrial biofilms across distant geographic regions and climate zones. However, there is little existing molecular evidence to address the question of microbial biogeography in terrestrial Green algae. With focusing on a few exemplar morphogenera, we aim to investigate the widely accepted idea of cosmopolitan distribution of terrestrial Green microalgae.

Summary of the Chapters

Within the frame of this thesis, terrestrial samplings from two main sources were examined. Soil samples originating from 27 grassland and 30 forest sites of the German Biodiversity Exploratories were investigated in **Chapters 1, 3-4**. Semi-terrestrial biofilm samples originating from two karstic streams in Germany were studied in **Chapter 2**. Culturing and sequencing approach was applied consistently throughout the Chapters. Environmental samples were cultivated in standard enrichment media favoring the growth of terrestrial Green algae. After isolating the algae into monoclonal cultures, they were subjected to combined microscopical and molecular phylogenetic analyses. New sequence data were complemented by accessions from GenBank and additional algal strains were kindly provided by the SAG Culture Collection of Algae and by further projects of the Department of Experimental Phycology (Lepka 2007; Spitzer 2013).

Chapter 1 | *Molecular diversity of microscopic Green algae isolated from German soils*

Here we examined the diversity of culturable Green microalgae dwelling in forest and grassland soils. We asked whether our cultured strains genetically match SSU-amplicons (clones, pyrotag reads) from published culture-independent diversity surveys. Our approach enables to link operational taxonomic units from soil metagenomes with physical organisms. We contribute an extensive dataset of sequenced isolates from Central Europe (57 sampling sites, 188 sequenced monoclonal cultures, 61 monophyletic species of Green microalgae). We further inform about new species of soil microalgae and one novel lineage witihn the class Trebouxiophyceae. By putting geographic data for identical ITS2-ribotypes on the world map, we suggest long-distance dispersal of *Stichococcus*, *Chlorella* and *Klebsormidium*.

Chapter 2 | *Diversity of microscopic Green algae (Chlorophyta) in calcifying biofilms of two karstic streams in Germany*

Green algal biofilms cover periodically desiccating stromatolites in karstic streams in Germany. We investigated this unusual semi-terrestrial habitat for the first time using a combined morphological and molecular approach. Thirty-four species were inferred from 18S rDNA sequence analyses. Among the closest relatives are species known from aquatic and terrestrial habitats. We isolated filamentous Green algae morphologically resembling *Gongrosira* Kützing, often reported from freshwater tufa-stromatolites. Molecular analysis suggests that the taxon

represents a collective morphotype, which encompasses several genera phylogenetically nested within the Ulvophyceae (Chlorophyta).

Chapter 3 | *Phylogenetic analysis of polar Chlorella and Stichococcus suggests biogeography of airborne microalgae*

Here we aim to uncover biogeographic patterns in cosmopolitan morphospecies *Chlorella* and *Stichococcus*. Already published 18S rDNA sequences from terrestrial and aquatic habitats worldwide, provided a basis for biogeographic analyses. We expanded existing data by new sequences from terrestrial habitats of Germany, Ecuador, the Arctic and Antarctica. We found out that particular *Stichococcus* clades exhibit either temperate-polar or temperate-tropical distribution, but not both. The clades of *Chlorella*-like microalgae which include polar and hot desert strains (e.g., *C. vulgaris* and *Muriella terrestris*) were so far never uncovered in the tropics. Our data provide evidence that airborne microalgae might exhibit biogeography.

Chapter 4 | *Molecular evidence for the wide distribution of two lineages of terrestrial Green algae (Chlorophyta) from tropics to temperate zone*

Terrestrial microalgae *Jenufa* and *Xylochloris* were originally described from the Southeast Asia. Our discovery of similar 18S rDNA sequences in terrestrial samples originating from temperate Europe and South and Central America suggests temperate-pantropic distribution of *Jenufa* and *Xylochloris*. Both lineages occur in soils, but differ in subaerial growth; whereas *Jenufa* growths on rocks and artificial hard substrates, *Xylochloris* was so far detected on organic substrates (tree barks).

Chapter 1

Molecular diversity of microscopic Green algae isolated from German soils

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(Manuscript draft)

Abstract

Microscopic Green algae are highly diversified in soils of temperate forests and grasslands. The morphology-based literature counts hundreds of (morpho)species, however, only a minor fraction of this diversity has been verified via DNA sequencing of monoclonal cultures. Therefore, SSU-based operational taxonomic units in soil algal metagenomes can hardly be linked with physical organisms. Here we extended the sampling of sequenced algal isolates and cultivated soils from 57 sampling sites in Central European forests and grasslands. We isolated and sequenced 188 monoclonal cultures and retrieved 61 species of Green microalgae. More than a half of them match conspecific cultures isolated elsewhere in Europe, predominantly from terrestrial habitats, but also from freshwaters. Furthermore, 90% of our new cultures match environmental clones retrieved from the same sampling sites and from different terrestrial and aquatic habitats. We contribute new ITS2-based evidence of the long-distance dispersal of *Stichococcus*, *Chlorella* and *Klebsormidium*. On the other hand, at least seven novel species were detected, suggesting that soils in Central Europe might veil algal diversity hardly accessible without culturing.

Keywords: soil biodiversity; Chlorophyta; monoclonal cultures, phylogeny, biogeography, morphology

Abbreviations: MB, Bayesian inference; ML, maximum likelihood; ITS2, internal transcribed spacer 2; OTU, operational taxonomic unit; GBE, German Biodiversity Exploratories; HAI, Hainich; ALB, Schwäbische Alb; SCH, Schorfheide-Chorin

Introduction

Molecular diversity of soil microorganisms is far from well understood (Daniel 2005; Mocali and Benedetti 2010; Shade *et al.* 2012). Nonetheless, investigations of soil prokaryotes successfully target ecological questions (Lauber *et al.* 2009; Nacke *et al.* 2011; Leff *et al.* 2015; Prober *et al.* 2015), accelerated by modern diversity acquisition techniques. In contrast, less attention was paid to the molecular diversity of soil eukaryotic photoautotrophs—microalgae abundant in the topsoil horizon (Starks *et al.* 1981; Hoffmann *et al.* 2007; Wojtuń *et al.* 2013) and generally recognized as primary producers and soil stabilizers (Lukešová 2001; Hu *et al.* 2002; Hu *et al.* 2003a; Hu *et al.* 2003b). Unlike in the case of bacteria (Preston-Mafham *et al.* 2002; Prosser 2002) and fungi (Bailly *et al.* 2007; Prober *et al.* 2015) functional diversity of eukaryotic microalgae in soils remains poorly understood. The prevalent morphology-based concept of ecologically meaningful algocenoses (Gollerbach and Shtina 1969; Zenova *et al.* 1995; Kuzyakhmetov 1998; Şalaru *et al.* 2008; Kabirov *et al.* 2010) urgently needs molecular re-evaluation. Similar to prokaryotes and non-photoautotrophic protists (Boenigk et al. 2012; Caron et al. 2009; Couradeau et al. 2011), molecular diversity of microalgae is underestimated (Guiry 2012; Škaloud *et al.* 2015). Despite considerable sequencing efforts focused on microalgae and other protists in aquatic ecosystems (Moon-van der Staay *et al.* 2001; Chariton *et al.* 2010; Cheung *et al.* 2010; Medinger *et al.* 2010), molecular diversity of soil microalgae remains poorly explored (Lawley *et al.* 2004; Bates *et al.* 2013). Estimates of species richness in soils significantly differ between culture-dependent and culture-independent approaches. This applies for prokaryotes (Rondon *et al.* 2000; Daniel 2005; Shade *et al.* 2012), non-photoautotrophic protists (Moon-van der Staay *et al.* 2006; Doherty *et al.* 2007; Lara *et al.* 2007) and terrestrial microalgae (Hallmann *et al.* 2009; Hallmann *et al.* 2013a; Spitzer 2013). Guiry (2012) estimates that there are approximately 72 500 algal species, 13 000 of these species represent Chlorophyta with 8000 described (cultured) species and 5000 species to be discovered. Even though this estimate relies on Algaebase—the largest online database of information on algae (Guiry and Guiry 2015)—species were verified through molecular methods in a minority of cases.

18S rDNA (SSU) marker once changed our view of microbial diversity (Moreira and Lopez-Garcia 2002), was substantially criticized (Forney *et al.* 2004; Richards and Bass 2005; Škaloud *et al.* 2015), but still holds its significance due to application universality (Lesaulnier *et al.* 2008; Bråte *et al.* 2010; Cheung *et al.* 2010; Bates *et al.* 2013; Lutz *et al.* 2015). However, culture-independent diversity studies often miss a deeper taxonomic resolution and treat Green microalgae as undifferentiated group—Chlorophyta (Lawley *et al.* 2004; Brown *et al.* 2009;

Bates *et al.* 2013; Lutz *et al.* 2015). Number of detected species (or operational taxonomic units, OTUs) directly depends from sequence-similarity threshold-value and sequence length (Caron *et al.* 2009; Grattepanche *et al.* 2014). Eukaryote-wide next generation sequencing (NGS) studies prefered SSU hypervariable regions SSU-V4 (Cheung *et al.* 2010; Taib *et al.* 2013) and SSU-V9 (Amaral-Zettler *et al.* 2009; Taib *et al.* 2013). Considering SSU-V4, 97% sequence similarity is a widely accepted minimum cutoff-value, applied in e.g. Ion Torrent sequencing of snow Chlorophyta (Lutz *et al.* 2015), 454-pyrosequencing of Chlorophyta in an estuary reservoir (Sun *et al.* 2014) or from saltern ponds (Filker *et al.* 2015). The maximum cutoff-value is usually 99%, used in e.g. 454-pyrosequencing of aquatic Chlorophyta (Zhan *et al.* 2013). In biodiversity acquirements, next generation sequencing outperform any traditional approach by means of achieved species/OTU quantity and detection of extremely rare and (so far) uncultivable species (Shi *et al.* 2009; Santoferrara *et al.* 2014; Xiao *et al.* 2014). On the other hand, Xiao *et al.* (2014) noticed limits of NGS for microalgal species resolution and affirmed importance of microscopic species identification. The same recognized Bazin *et al.* (2014) applying 98% cutoff and Viprey *et al.* (2008), who proposed a more stringent threshold for species identification (98.5-99.5%), both cloning marine Green microalgae.

In the taxonomy of Green microalgae, cultures are crucial for precise species characterization (Lukešová 1993; Ettl and Gärtner 1995; Lukešová and Hoffmann 1996; Lukešová 2001). Fine scale identification of microorganisms is essential for understanding their role in ecosystems (Rastogi and Sani 2011) and urgently required for reproducible comparisons of biodiversity shifts between environments (Richards and Bass 2005). Cultures of microalgae are not only valuable for original species characterization, but also for preservation of genetic material (Müller *et al.* 2007a; Day *et al.* 2010). This applies particularly in times of accelerated biodiversity loss (Pimm and Raven 2000). Monitoring of microbial/protistan biodiversity shifts correlated with climate change will hardly be possible without preservation of the current status, e.g., by maintaining cultures or environmental samples (Cary and Fierer 2014; Fierer and Cary 2014). Leading morphology-based identification key for terrestrial algae counts 785 species of (mostly cultured) Green microalgae (Ettl and Gärtner 1995; Ettl and Gärtner 2014). It is not clear how the (morpho)species diversity correlates with OTU-based diversities reported from terrestrial ecosystems via culture-independent techniques. This is due to scarcity of biodiversity surveys combining morphological observations and molecular techniques. Such comparative studies so far focused mainly on phytoplankton (Krienitz and Bock 2012), semi-terrestrial biofilms (Brinkmann et al. 2015; Hodač et al. 2015) and aerophytic biofilms (Lin *et al.* 2012; Kulichová *et al.* 2014). Regarding soils—the most species-rich terrestrial habitats—polyphasic data are

available so far for arid lands (Lin and Wu 2014; Flechtner et al. 2013; Fučíková et al. 2014) or subtropical farmlands (Lin *et al.* 2013).

In the present study, for the first time we investigate molecular diversity of culturable Green microalgae in soils from Central Europe. The soils of this region remain almost untouched by culture-dependent molecular diversity surveys targeting Green microalgae, despite the fact that significant fraction of them were described from here (Ettl and Gärtner 1995). Expecting differences in cultured and uncultured microbial diversities, we hypothesize that extensive sampling and culturing would retrieve new species, not yet submitted to GenBank from culture-independent surveys. We investigate 188 new monoclonal cultures of soil Green microalgae sampled from 57 sites covering deciduous and needle forests and meadows and pastures in Germany (Fischer *et al.* 2010). To achieve comparability with culture-independent studies, we sequenced SSU marker and conducted species identification following monophyletic species concept (Johansen and Casamatta 2005; Mallet 2005; Vyverman *et al.* 2010; Škaloud and Rindi 2013; Leliaert *et al.* 2014). Allowing minimal sequence divergence—using high sequence similarity threshold of ≥ 99.90%—we aim to evaluate how our data match already published cultured species. In a second step we test whether our cultured species match OTU-based diversities acquired by cloning or NGS techniques, hereby applying a similarity threshold of 97% for pyrotags (Sun *et al.* 2014; Lutz *et al.* 2015) and 99% for clones (Viprey *et al.* 2008; Zhan *et al.* 2013). Since soil is a semi-aquatic habitat, it presumably hosts an assemblage of terrestrial and aquatic species. Though morphological literature suggest cosmopolitanism of microalgae in temperate soils (Ettl and Gärtner 1995; Lukešová 2001), the molecular evidence is missing. This we aim to prove by comparing our isolates with cultures sampled elsewhere, analyzing the highly variable ITS2 marker (Müller *et al.* 2007b; Coleman 2009; Buchheim *et al.* 2011; Keller 2011; Caisová *et al.* 2013; Wolf *et al.* 2013).

Material and Methods

Sampling sites

Soil samples were collected from defined plots within large-scale and long-term research sites termed Biodiversity Exploratories (Hainich-Dün, Abbr. HAI; Schorfheide-Chorin, Abbr. SCH; Schwäbische Alb; Abbr. ALB; **Fig. 1a**). The sampling covers plots in forests (**Figs. 1b, 1c, 1d**) and grasslands (**Figs. 1e, 1f**), which represent a gradient of land-use intensities, described in detail by Fischer *et al.* (2010). We analyzed samples from two soil horizons: 1) drill core (A-horizon) samples were collected in HAI, SCH, ALB in April 2008 from 27 grassland and 30 forest plots

and multiple samples from within each plot were pooled, 2) soil surface (O-horizon, upper 3 cm) samples were taken in autumn (September 2010) and spring (March 2011, June 2011) from two forest and three grassland plots within the HAI exploratory (using a sterile spoon and transported in sterile Petri dishes. In the latter case, three different sampling points were defined within each plot (middle, north-east corner, south-west corner; **Fig. S5e**). These subplot samples were analyzed in two ways, i.e., separately and pooled together.

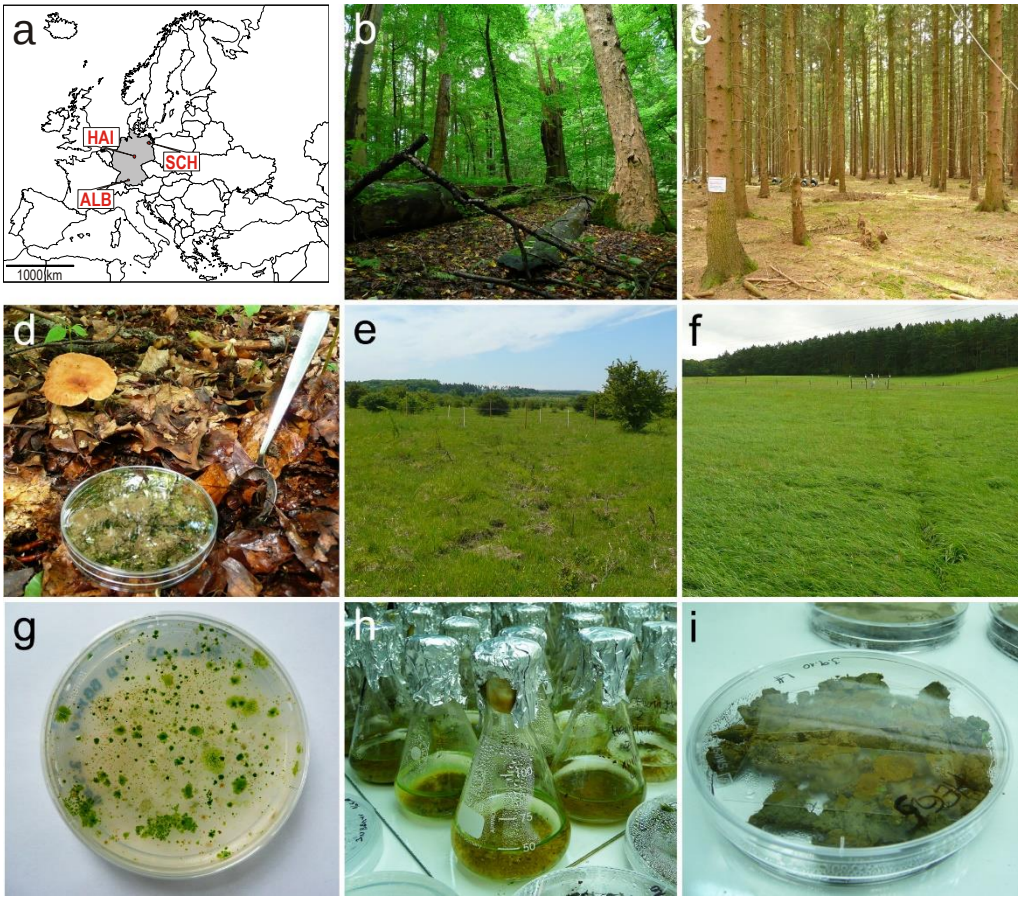

Figure 1. Soil sampling in the German Biodiversity Exploratories. (a) Localization of the three sampling areas (highlighted in red): ALB=Schwäbische Alb, HAI=Hainich-Dün, SCH=Schorfheide-Chorin). (b)-(c) Examples of sampling sites in extensively managed (b) and intensively managed (c) forests (Hainich). (d) Soil sample taken by a spoon from topsoil. (e)-(f) Examples of sampling sites in extensively managed (e) and intensively managed (f) grasslands (Hainich). (g)-(i) Culturing techniques–establishment of mixed cultures. (g) Algal colonies on agar plate with spread soil suspension); (h) Algal growth in soil suspended in liquid BBM medium (Bischoff and Bold 1963); (i) Culturing of microalgae on coverslips put on the soil surface.

Soil culturing, isolation and light microscopy of monoclonal cultures

Two grams of each soil sample were suspended and cultured in liquid media and spread on agarized media (**Fig. 1g**). The composition of liquid (in 100 ml Erlenmeyer flasks; **Fig. 1h**) and 1.5% agarized (Petri dishes) media was the same: 3NBBM+V and BG11 (EPSAG; https://www.uni-goettingen.de/de/list-of-media-and-recipes/186449.html). Both liquid and

agarized cultures were kept for at least six weeks at 18 °C under a light : dark regime of 14 : 10 hours at a light intensity of about 25 µE from a white fluorescent bulb. After three, four and six weeks of growth in liquid media, algal suspension was inspected using a light microscope. The algal colonies growing on the agarized plates were transferred with sterile inoculating loops onto new fresh plates with the same medium and final monoclonal cultures were kept on agar slants (1.5%) under the same growth conditions. The soil surface samples were treated in the same way as described above. The only modification concerns the BG11 medium which was replaced by Z medium. In addition to the culturing techniques on media, cover slips method was used (Ettl and Gärtner 1995; Lukešová 2001). Sterile glass cover slips were put directly on wet soil samples (stored in sterile Petri dishes; **Fig. 1i**) and after 2-3 weeks of algal growth on the contact surface, the covers slips were analyzed by light microscopy. The light microscopy of the mixed raw cultures and unialgal isolates was accomplished using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics with an attached ColorView III camera (Soft Imaging System, Münster, Germany). The observations were documented by micrographs, processed using the Cell^D image software (Soft Imaging System, Münster, Germany). Cell size measurements (diameter of spherical cells; length/width of elongated cells) were conducted were conducted in program ImageJ (Schneider *et al.* 2012) and were based on 100 cells per culture. and the summary statistics including maximum and minimum values were computed in PAST 2.12 (Hammer *et al.* 2001).

DNA isolation, PCR and sequencing

DNA was extracted from 188 monoclonal isolates (**Table S1**) using the Invisorb Spin Plant Kit (Stratec molecular, Berlin, Germany) as recommended by the manufacturer. 18S rDNA marker was amplified using primers NS1 and 18L (Hamby *et al.* 1988). For selected isolates 18S and ITS2 rDNA gene region was amplified using primers NS1 and LR1850 (Friedl 1996). Conditions for PCR and sequencing reactions and the standard set of sequencing primers were described previously by Mikhailyuk *et al.* (2008). The newly determined sequences will be deposited in GenBank. Two additional isolates from polar regions (Shukla *et al.* 2011), and two from Ecuador (Spitzer 2013; Faßhauer in prep.) were sequenced for 18S rDNA.

Phylogenetic analysis

The newly obtained 80 full to almost full 18S rDNA sequences (~1600-1800 nucleotides in length; **Tables S2a**, **S2b**) were compared to NCBI database using BLAST queries (Altschul *et al.*

1997) in order to download all closest relatives available from GenBank (Benson *et al.* 2012). All sequences were checked for chimeras by Bellerophon (Huber *et al.* 2004). Two alignments were established, one for Green algae (Chlorophyta + Streptophyta) and another for Stramenopiles including Xanthophyceae and Eustigmatophyceae. Both alignments were further processed in the same way. Alignments were computed by the program MAFFT-Q (Katoh and Toh 2008) and inspected in BioEdit (Hall 1999) for possibly misaligned nucleotide positions. The alignment of Green algae consisted of 169 sequences with 1817 positions (711/564 variable/parsimony informative). The alignment of Stramenopiles consisted of 72 sequences with 1895 positions (613/434 variable/parsimony informative). In both cases, the GTR+Γ+I model was selected as the best fitting substitution model using AIC criterion in jModelTest 0.1.1 (Posada 2008). Phylogenetic trees were calculated using maximum likelihood method in the program RAxML 7.0.4 (Stamatakis *et al.* 2008). The Bayesian phylogenetic trees were calculated using MrBayes 3.2.1 x64 (Ronquist *et al.* 2012). For the latter, two MCMC runs for three million generations each were employed with one cold and three heated chains with trees sampled every 100 generations. Internal edge support values were inferred from the rapid bootstrapping algorithm (100 replicates) as implemented in RAxML (Stamatakis *et al.* 2008) and from Bayesian posterior probabilities using MrBayes 3.2.1 x64 (Ronquist *et al.* 2012). Pairwise sequence similarities were derived from p-distances based on Kimura-2-parameter model computed in the program MEGA5 (Tamura *et al.* 2011). Pairwise similarities among our isolates and their closest GenBank relatives are listed in **Table S3**. The same method was used to identify species represented by partial 18S rDNA sequences (**Tables S4a**, **S4b**).

Delimitation of morphotypes and statistical analysis of their distribution

The morphological diversity of monoclonal cultures is summarized in **Table S5**. Observations on morphotypes occurring in mixed cultures (agar plates, liquid media and cover slips) were documented as binary presence/absence matrices (**Table S6a**; **Table S6b**) and summarized in **Table S7**. Delimitation of morphotypes was accomplished by identification literature (Reisigl 1964; Trenkwalder 1975; Metting 1981; Huber-Pestalozzi *et al.* 1983; Ettl and Gärtner 1995; Andreyeva 1998; Komárek and Anagnostidis 1998; Komárek and Anagnostidis 2005; Hoffmann *et al.* 2007; Škaloud 2009; Komárek 2013; Ettl and Gärtner 2014). Morphotype representatives are documented as drawings of monoclonal cultures and microphotographs of monoclonal cultures and mixed cultures. Where it was possible, taxa were morphologically determined at the genus level. Otherwise, assignation at higher taxonomic level was preferred. Taxonomically

ambiguous taxa predominantly occurring as similar living forms were combined into collective morphotypes. For example, sarcinoid forms traditionally named *Chlorosarcina*, *Chlorosarcinopsis* and *Desmotetra* represented one collective morphotype and named Chlorosarcinaceae. The resulting binary presence/absence matrix was further used for multivariate statistical analysis. Prior to the analysis, we pooled the presence/absence data of each three replicates representing the same land-use intensity, e.g., AEG1+AEG2+AEG3=AEG_i (intensively managed grassland plots within ALB). Redundancy analysis (RDA) (Hill 1979; Jaarsma *et al.* 2006) was conducted to test for correlations between important soil physico-chemical parameters, i.e., pH, organic carbon/C_{org}, total nitrogen/N_{tot} (Metting 1981; Marschner *et al.* 2003) and the composition of algal morphotypes at the sampling sites. The original measurement data were obtained from Nadine Herold in the frame of the German Biodiversity Exploratories project. Because the diversity data were pooled within each land-use category (Fischer *et al.* 2010), in the ordination analysis we used corresponding arithmetic means of each physico-chemical parameter. For statistical support the Monte Carlo test with 2000 permutations under reduced model was used (Hope 1968). The morphotype composition of the soil surface samples was analyzed using Non-metric Multidimensional Scaling (NMDS) using Jaccard distance measure. All statistical analyses were performed in PAST version 2.12 (Hammer *et al.* 2001) and CANOCO version 4.5 (ter Braak and Šmilauer 2002). Hence Green algae (Chlorophyta, Streptophyta) and stramenopiles (Xanthophyceae, Eustigmatophycae) were present in almost all samples and many taxa are relatively easy to cultivate (compared to e.g. Diatoms or Cyanobacteria) we focused on them in further molecular studies.

Results

Molecular diversity and morphology of the monoclonal isolates

We analyzed a total of 188 18S sequences; 146 sequences were longer (1600-1800 bp) and 42 shorter (300-1600 bp). Within this dataset we distinguished different 73 phylotypes corresponding to species at ≥ 99.90% sequence similarity level. The species belong mainly to Green algae: Chlorophyta (59 spp.) and Streptophyta (2 spp.) and to Stramenopiles: Xanthophyceae (11 spp.) and Eustigmatophyceae (1 sp.).

Green algae

We detected 61 species assigned of 21 lineages from four classes of Chlorophyta (Chloro-, Trebouxio-, Ulvo- and Prasinophyceae) and one class of Streptophyta (Klebsormidiophyceae).

The Chlorophyceae are represented by ten lineages: *Oogamochlamydinia, Chloromonadinia, Tatrensinia, Reinhardtinia, Desmotetra, Stephanosphaerinia, Jenufa, Scenedesmaceae, Pseudomuriellaceae, Bracteacoccaceae*). The Trebouxiophyceae are represented by eight lineages: *Prasiola/Stichococcus*, *Neocystis*, *Botryococcus*, *Watanabea*, *Lobosphaera*, *Xylochloris*/*Dictyochloropsis*, *'Navichloris'*, Chlorellaceae. The Ulvophyceae are represented by Ulotrichales and Pedinophyceae by *Pedinomonas*. The Klebsormidiophyceae is represented by *Klebsormidium*.

Streptophyta; Klebsormidiophyceae

The Streptophyta were represented by two different species of *Klebsormidium* (**Fig. 2**), common terrestrial algae with unbranched filaments and a single band-shaped chloroplast (with a pyrenoid) per cell. (I) *Klebsormidium flaccidum* (e.g., isolate LH10HG2056; **Fig. 3a**) from grasslands (HAI, SCH) and forest (HAI) is identical to *K. flaccidum* SAG 7.91 (EU434019) from freshwater (**Fig. 2**). *Klebsormidium* cf. *dissectum/elegans* (isolate LH08HW9106; **Fig. 3b**) from grasslands (ALB) and forests (HAI) is identical to authentic strain *K. dissectum* SAG 2155 (from soil) and to authentic strain *K. elegans* SAG 7.96 (from tree bark). The whole clade including species of *Klebsormidium* and *Interfilum* is highly supported.

Chlorophyta; Pedinophyceae

Prasinophyte algae were represented by one isolate belonging to *Pedinomonas* (**Fig. 2**), exhibiting small *Chlamydomonas*-like cells containing one pyrenoid and basal stigma. *Pedinomonas* cf. *minor* (isolate LH08SG2033; **Fig. 3c**) from grassland (SCH) is closely related to *P. minor* SAG 1965-3 (HE610132) from freshwater (**Fig. 2**). The clade comprising both accessions is highly supported.

Chlorophyta; Ulvophyceae

The class Ulvophyceae is here represented by the Ulvales/Ulotrichales lineage (Watanabe and Nakayama 2007) and one genus *Pseudendocloniopsis* (**Fig. 2**), with spherical to ovoid (slightly acuminate) cells with a cup-shaped chloroplast and prominent pyrenoids. *Pseudendoclonium* cf. *botryoides* (isolate LH08HW9058; **Fig. 3d**) from forest (HAI) was closely related to authentic strain *P. botryoides* SAG 465-1 (AJ416103) from freshwater. The clade comprising both accessions is highly supported.

Chlorophyta; Chlorophyceae; Sphaeropleales.

Scenedesmaceae (Hegewald and Hanagata 2000) comprised three species (**Fig. 2**) with spherical to symmetrically elliptic cells with slightly acuminate poles. (I) *Coelastrella multistriata* (e.g., isolate LH10HG7083; **Fig. 3e**), identical to terrestrial *C. multistriata* Hanagata C6-2 (AB012846), was found in grasslands (HAI, ALB) and forest (ALB). (II) *Coelastrella* sp. (e.g., isolates LH10HG2098, LH10HG7018; **Fig. 3f**) occurs in grasslands (HAI) and is identical to *Scenedesmus* sp. KGU Y002 (AB742453). (III) *Acutodesmus rubescens* (e.g., isolate LH08SG8041; **Fig. 3g**) from grassland (SCH) is identical to *'Scenedesmus' (Acutodesmus) rubescens* CCAP 232/1 (X74002). The Scenedesmaceae clade is highly supported.

Members of Pseudomuriellaceae and Bracteacoccaceae (Fučíková *et al.* 2013) exhibit spherical cells with several to multiple parietal chloroplasts without pyrenoids. *Pseudomuriella aurantiaca* (isolates LH10HG9038, LH10HG2039; **Fig. 3h**) from grasslands (HAI) is identical to the authentic strain *P. aurantiaca* SAG 249-1 (X91268) isolated from soil. *Bracteacoccus cohaerens* (isolate LH10HG9034; **Fig. 3i**) from grassland (HAI) is identical to the authentic strain *B. cohaerens* UTEX 1272 (GQ985406) from soil (USA). Another isolate from grassland (SCH), *Bracteacoccus* cf. *cohaerens* (isolate LH08SG2015), is slightly different from the same GenBank accession but might be as well conspecific. Both *Pseudomuriella* and *Bracteacoccus* clades were highly supported.

Chlorophyta; Chlorophyceae; Chlamydomonadales

Isolates assigned to Chlorophyceae were nested in two major lineages Sphaeropleales and Chlamydomonadales, and *Jenufa* clade incertae sedis (**Fig. 2**). The Chlamydomonadales isolates were characteristic for monadoid/flagellate *Chlamydomonas*-like and/or coccal *Chlorococcum*-like morphologies (**Fig. 4a-k**). The Sphaeropleales were either coccal colonial *Scenedesmus*-like, or non-colonial *Bracteacoccus*-like (**Fig. 4a-e**).

Oogamochlamydinia clade (Nakada *et al.* 2008). The clade comprises monadoid isolates belonging to two species of similar morphology differing in cell size. (I) *Oogamochlamys* sp.(I) (isolate LH08SG8047; **Fig. 4a**) from grassland (SCH) is less closely related to *O. ettlii* UTEX 2218 and *O. gigantea* SAG 21.72. (II) *Oogamochlamys* sp.(II) strain SAG 2476 (= isolate LH08AW1069); **Fig. 4b**) from forest (ALB) is less closely related to freshwater *Chlamydomonas* sp. CCAP 11/159. Both species are nested in well supported clade including *Oogamochlamys* and *Lobochlamys* accessions.

Chloromonadinia clade (Nakada *et al.* 2008). One monadoid species, *Chlamydomonas* cf. *gerloffii* (isolate LH08SW5031; **Fig. 4c**), found in forest (SCH), is closely related to freshwater

C. gerloffii CCAP 11/72 (FR865610). The species is nested in a highly supported clade including authentic strains *C. gerloffii* CCAP 11/72 and *Chloromonas rosae* UTEX 1337 accessions.

Reinhardtinia clade (Nakada *et al.* 2008). Monadoid isolates of *Chlamydomonas*-like morphology clustered in four different species. (I) *Chlamydomonas rapa* (isolates LH08SG1077, LH08SG9055) detected in grasslands (SCH), is identical to freshwater authentic strain *C. rapa* SAG 48.72 (U70790). (II) *Chlamydomonas* cf. *rapa* (isolate LH10HG1027; **Fig. 4d**) from grassland (HAI), is very closely related to *C. rapa* SAG 48.72 and might be conspecific as well. These three accessions create a highly supported clade, a sister group to *C. reinhardtii* (M32703). Two isolates from grasslands (ALB/SCH) were nested in another highly supported subclade within *Reinhardtinia* clade. (III) *Heterochlamydomonas* sp. (isolate LH08AG2004; **Fig. 4e**) closely related to freshwater *H. rugosa* SAG 45.86 (AF367859) and authentic strain *H. inaequalis* UTEX 1705 (AF367857). (IV) *Chlamydomonas* cf. *typica* (isolate LH08SG9022; **Fig. 4f**) is closely related to soil *C. typica* NIES-2246 (AB701557). The clade including the four species from Germany is highly supported.

Desmotetra clade (Nakada *et al.* 2008). *Desmotetra* Deason & Floyd detected in this study is characterized by pairs of widely elliptic non-motile cells remaining attached after division. *D. stigmatica* (isolate LH08SG2049; **Fig. 4g**) from grassland (SCH) is identical to authentic strain *D. stigmatica* UTEX B 962 (DQ009760) in a highly supported clade – a sister group of the *Reinhardtinia* clade. The species was further found in grassland (HAI; isolate LH10HG6P18).

Tatrensinia clade (Nakada *et al.* 2008). The clade comprises two *Chlorococcum*-like species from grasslands (HAI/SCH). (I) *'Tatrensinia' ('Chlorococcum')* sp.1 (LH08SW7115; **Fig. 4h**) is closely related to soil Haematococcaceae clone Amb_18S_582 (EF023273). (II) *'Tatrensinia' ('Chlorococcum')* sp.2 (isolates LH10HG7016, LH10HG9131; **Fig. 4i**) is closely related to *'Chlorococcum'* cf. *tatrense* (= *Chlamydocapsa* sp.) CCCryo 101-99 (AF514407) from Spitsbergen permafrost. Both species are nested in a highly supported clade.

Stephanosphaerinia clade (Nakada *et al.* 2008). Isolates exhibiting monadoid *Chlamydomonas*-like morphology clustered in four different species. (I) *Chlorococcum sphacosum* (*'Neospongiococcum' gelatinosum*) (isolate LH10HG3113; **Fig. 4j**) is identical to authentic strain *C. sphacosum* SAG 66.80 (JN968580) and authentic strain *N. gelatinosum* SAG 64.80 (JN968584), both isolated from soils. (II) *Chlamydopodium vacuolatum* (isolate LH10HG1013) identical to *'Chlorococcum' robustum* Kr 1986/30 (AY122332) from freshwater. (III) *'Stephanosphaerinia'* (*'Chlorococcum'*) sp. (isolate LH10HG6108), is closely related to freshwater isolate Chlorococcales sp. VII3 (FJ946904) from Antarctica. The three species are nested in a highly supported clade. Another highly supported clade consisted of (IV)

Chlorococcum cf. *minutum* SAG 2479 (= LH08AW5056; **Fig. 4k**) and closely related *C. minutum* SAG 21.95 (JN968585) from soil. *Chlorococcum* cf. *minutum* was detected in grassland and forest (ALB).

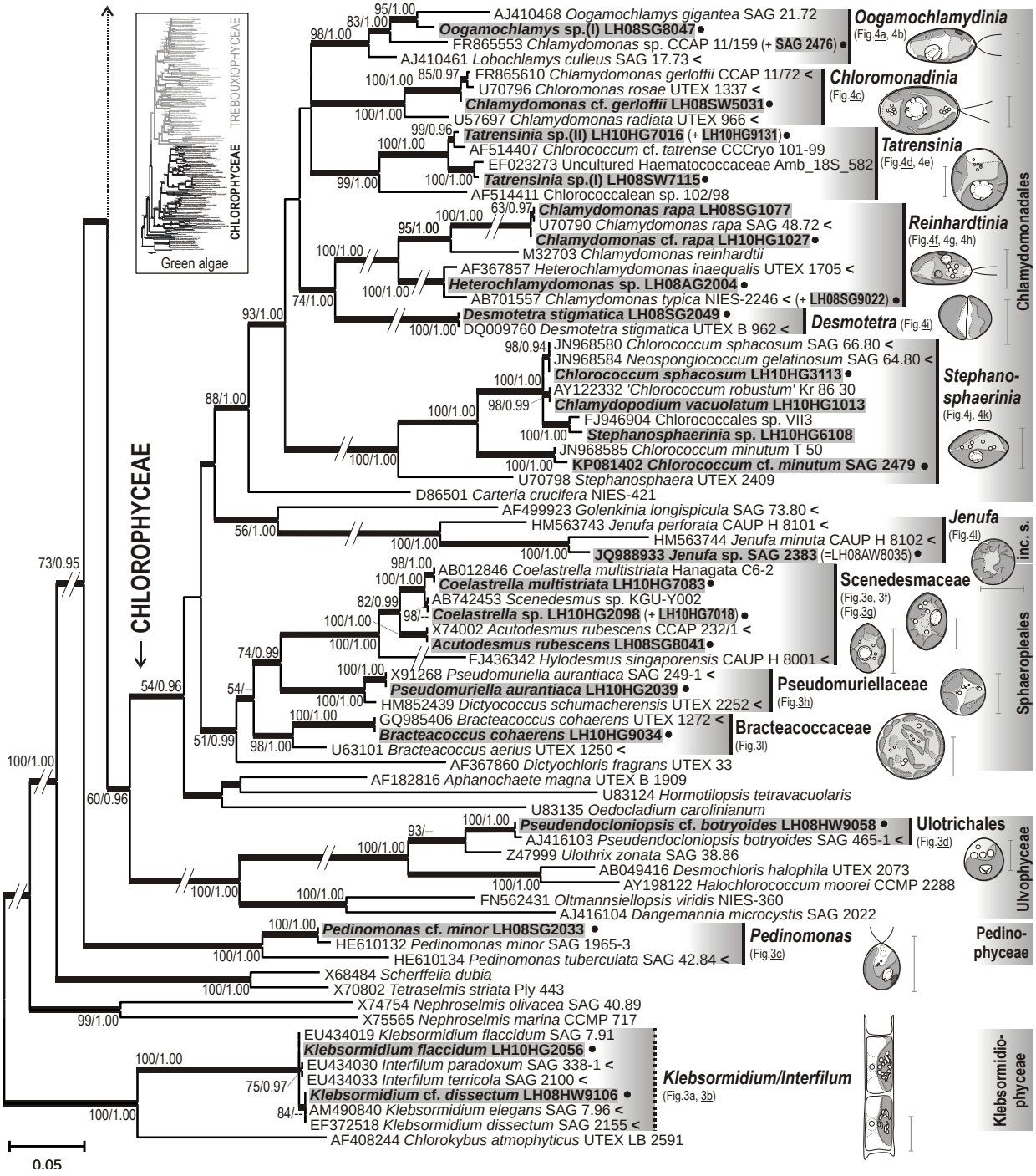

Figure 2. 18S rDNA phylogeny of Green algae, part 1 including Chlorophyceae, Ulvophyceae, Pedinophyceae and Klebsormidiophyceae. New accessions are written in bold and grey underlaid. Black dots mark references to microphotographs (**Fig. 3**, **Fig. 4**) sorted in top down order. Original drawings of representative morphologies are shown for each clade (scale bars = 5 µm). Sequences of authentic strains are marked by a '<' sign. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities).

Chlorophyta; Chlorophyceae; *Jenufa* clade

Jenufa clade (Němcová *et al.* 2011) comprises coccid species with spherical cells with an extensively lobed parietal chloroplasts without pyrenoids. *Jenufa* sp. SAG 2383 (= LH08AW8035; **Fig. 4l;** JQ988933) and (identical) isolate LH08AW8098, found in forest (ALB), are related to authentic strain *J. minuta* CAUP H 8102 (HM563744) from tree bark in Singapore. The *Jenufa* clade, incertae sedis within the Chlorophyceae, consists of *J. minuta* CAUP H 8102, *J. perforata* CAUP H 8101 and *Jenufa* sp. SAG 2383 (= LH08AW8035) and is highly supported.

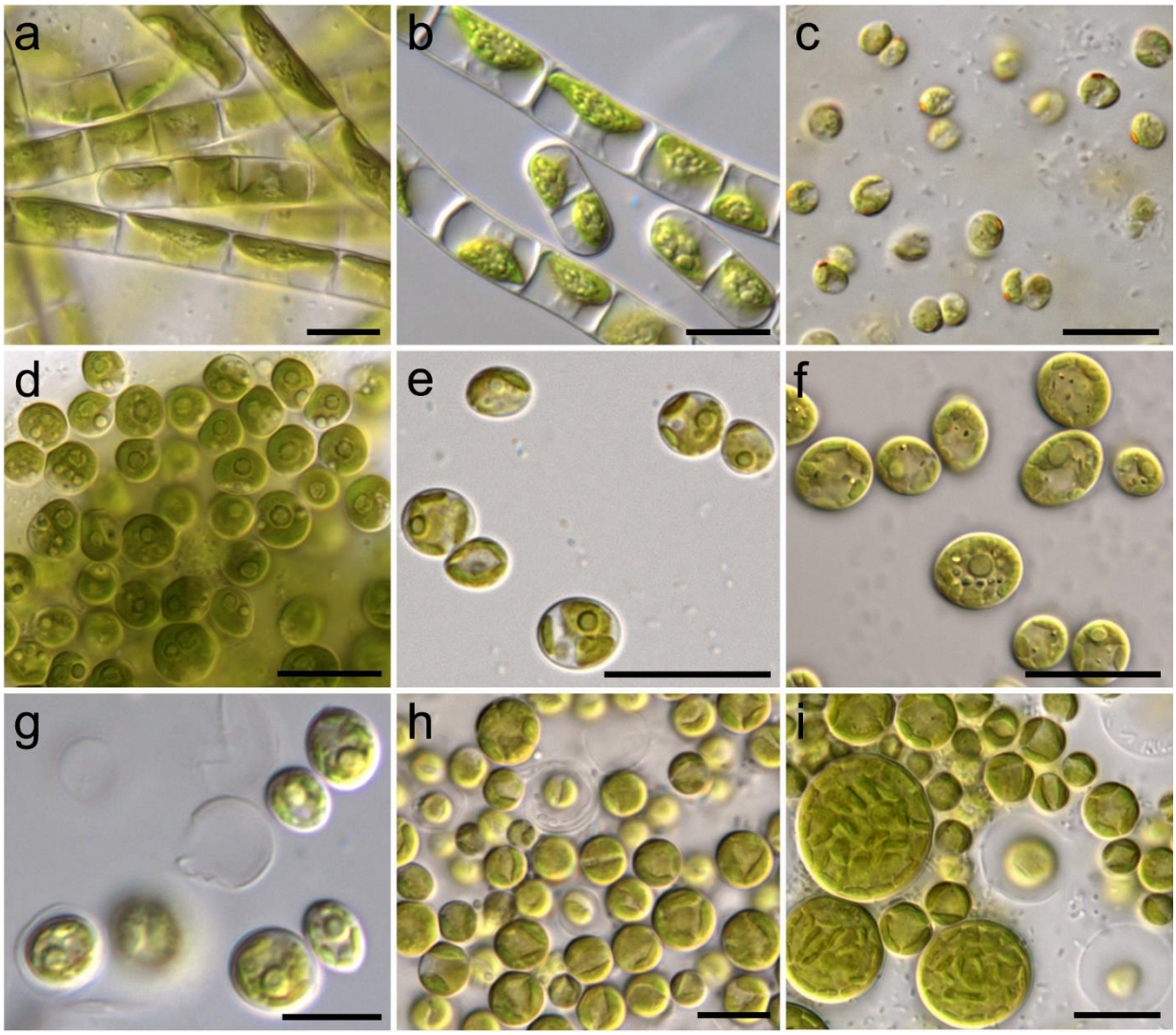

Figure 3. Microphotographs of monoclonal cultures of Green algae (Klebsormidiophyceae, Prasinophyceae, Ulvophyceae and Chlorophyceae/Sphaeropleales). (a) *Klebsormidium* cf. *flaccidum* LH10HG2056 (l=6.4-19.7 µm, w=4.5-7.0 µm); (b) *Klebsormidium* cf. *dissectum* LH08HW9106; (c) *Pedinomonas minor* LH08SG2033 (l=2.9-5.6 µm, w=2.5-4.8 µm); (d) *Pseudendocloniopsis botryoides* LH08HW9058 (Ø=4.7-9.5 µm); (e) *Coelastrella multistriata* LH10HG7083 (Ø=3.5-7.0 µm); (f) *Coelastrella* sp. LH10HG7018; (g) *Acutodesmus rubescens* LH08SG8041; (h) *Pseudomuriella aurantiaca* LH10HG2039 (Ø=3.6-8.2 µm); (i) *Bracteacoccus cohaerens* LH10HG9034 (Ø=5.4-12.3 µm). Scale bars = 10 µm.

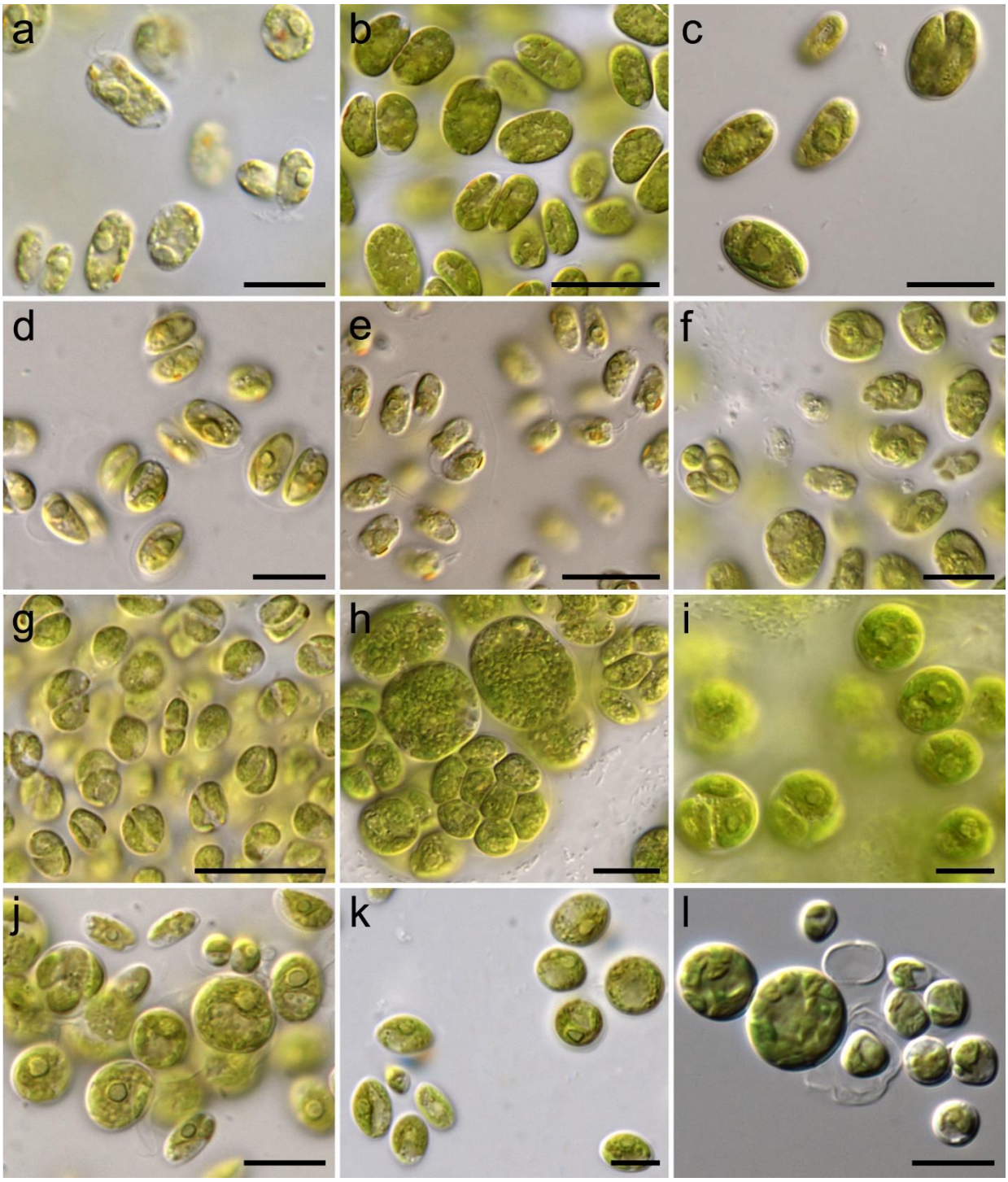

Figure 4. Microphotographs of monoclonal cultures of Green algae (Chlorophyceae/Chlamydomonadales). (a) *Oogamochlamys* sp.(I) LH08SG8047; (b) *Oogamochlamys* sp.(II) SAG 2476 (= LH08AW1069; l=9.1-14.5 µm, w=5.1-10.0 µm); (c) *Chlamydomonas* cf. *gerloffii* LH08SW5031 (l=10.9-16.4 µm, w=4.3-10.1 µm); (d) *Chlamydomonas* cf. *rapa* LH10HG1027 (l=6.1-9.9 µm, w=2.7-5.1 µm); (e) *Heterochlamydomonas* sp. LH08AG2004 (l=6.0-10.5 µm, w=3.1-7.5 µm); (f) *Chlamydomonas* cf. *typica* LH08SG9022 (l=6.7-12.2 µm, w=4.0-8.0 µm); (g) *Desmotetra stigmatica* LH08SG2049 (Ø=3.5-6.6 µm); (h) *Tatrensinia* sp.(I) LH08SW7115 (l=8.3-17.8 µm, w=5.2-13.3 µm); (i) *Tatrensinia* sp.(II) LH10HG9131 (Ø=7.9-13.1 µm); (j) *Chlorococcum sphacosum* LH10HG3113 (l=5.8-9.3 µm, w=2.6-6.3 µm); (k) *Chlorococcum* cf. *minutum* SAG 2479 (= LH08AW5056; l=5.6-9.5 µm, w=5.6-9.5 µm); (l) *Jenufa* sp. SAG 2383 (= LH08AW8035; Ø=2.9-7.3 µm). Scale bars = 10 µm.

Chlorophyta; Trebouxiophyceae

Isolates assigned to Trebouxiophyceae are phylogenetically nested in eight different clades (**Fig. 5**) and comprised rod-shaped, spherical and elliptic morphologies (**Fig. 6**).

Stichococcus-like rod-shaped isolates were spread across the *Prasiola/Stichococcus* clade (Sluiman and Guihal 2008) and we distinguished at least nine different species. (I) *'Stichococcus'* sp. (isolate LH08AW8025; **Fig. 6a**) from forest (ALB) is identical to *'Stichococcus'* sp. SAG 2059 (AY762604) from a building façade. (II) *'Stichococcus'* sp. (isolate LH08SG5057; **Fig. 6b**), occurring in grasslands (HAI, SCH, ALB), is identical to *'Stichococcus'* sp. SAG 2060 (AY762606) from a building façade. These species clustered near to authentic strain *Stichococcus deasonii* UTEX 1706 (DQ275460) from soil, yet the clade is not supported. (III) *'Stichococcus'* sp. (isolates LH08AG7010, LH10HG6110) from grasslands (HAI, ALB) is closely related to authentic strain *Stichococcus deasonii* UTEX 1706 (DQ275460). (IV) *'Stichococcus'* sp. (isolate LH08SG1073) from grassland (SCH) is identical to *'Stichococcus'* sp. SAG 2406 (= WB47; KF144240) from Westerhöfer creek. (V) *'Stichococcus'* sp. (isolate LH08SW1099) from forest (SCH) clustered near to the aforementioned species (IV) in an unsupported clade. (VI) *'Stichococcus'* sp. SAG 2482 (= LH08AW8023; **Fig. 6c**) from forest (ALB) is identical to *'Stichococcus'* sp. D4-2A (KF144238) from Deinschwanger creek. (VII) *Stichococcus* sp. SAG 2481 (= LH08AW8002) from forest (ALB) is identical to *Stichococcus* sp. K4-4 (AB055866) of unknown origin (probably isolated in Japan). *Pseudostichococcus* is represented by two species exhibiting rod-shaped *Stichococcus*-like morphology. (I) *Pseudostichococcus* cf. *monalantoides* (e.g., isolate LH10HG3045), detected in grasslands (HAI, ALB), is closely related to authentic strain *P. monallantoides* SAG 380-1 (JX185690). (II) *Pseudostichococcus* sp. (isolate LH08SW8044) from forest (SCH) is identical to *'Stichococcus' mirabilis* CCAP 379/3 (AJ311638). The clade comprising both *Pseudostichococcus* species and *'Stichococcus' mirabilis* CCAP 379/3 consistently occurred in both maximum-likelihood and Bayesian phylogenies, yet without statistical support. *Diplosphaera* is represented by two species exhibiting spherical to slightly cylindrical (shortly rod-shaped) cells which characteristically remain in doublets after cell division. (I) *'Chlorella'* cf. *sphaerica* (isolate LH08HW8075) from forest (HAI) is very closely related to authentic strain *C. sphaerica* SAG 11.88 (AJ416105) from New Zealand. Both specimens might be conspecific. Another isolate (LH08AG9089) from grassland (ALB) is as well closely related to the same authentic strain with a slightly higher genetic distance. The clade including both species (*Diplosphaera* clade) is well supported.

Elliptic morphotypes. Elliptic cell forms were characteristic for phylogenetically unrelated lineages *Neocystis* (Eliáš *et al.* 2013), *Coccomyxa* (Darienko *et al.* 2015), *Chloroidium* (Darienko

et al. 2010) and *'Navichloris'* (this study). The isolates from grasslands (HAI, SCH, ALB) and forests (HAI, ALB) belonging to *Neocystis brevis* (e.g., LH10HG9080; **Fig. 6d**) are identical to authentic strain *N. brevis* CAUP D 802 (JQ920360) from soil (the *Neocystis* clade was highly supported). *Coccomyxa* was represented by three species. (I) *C. simplex* (isolate LH08SG9051; **Fig. 6e**) from grassland (SCH) is identical to authentic strain *C. rayssiae* SAG 216-8 (= UTEX 273; HQ317304) from freshwater. The clade further includes authentic strain *C. chodatii* SAG 216-2 (FJ648512) and is highly supported. (II) *Coccomyxa viridis* SAG 2483 (= LH08AW8039; **Fig. 6f**), from forest (ALB), is identical to lichenized *C. glaronensis* CCALA 306 (AM167525). The clade comprises as well authentic strain *C. mucigena* SAG 216-4 (FJ648513) and is highly supported. (III) *Coccomyxa* sp. (isolate LH08AW1017) from forest (ALB) is very closely related to lichenized *Coccomyxa* sp. KN-2011-T3 (HE586515) from Indonesia. The clade additionally includes Uncultured Trebouxiophyceae RL75K2 (HE617184) from an acidic mining lake in Germany, and is highly supported. The strain SAG 2477 (= LH08AW3007; **Fig. 6g**; KP081399) detected in forest (ALB), exhibits symmetrically elliptic cells resembling *Coccomyxa* Schmidle. The species is neither phylogenetically related to *Coccomyxa* nor to other known genera and clades of Trebouxiophyceae. The closest BLAST-hit *Leptochlorella* sp. clone Qe17 (FJ790649) from China exhibits low similarity (= 95.54%). In both maximum-likelihood and bayesian phylogenies, SAG 2477 consistently clusters near to the *Leptochlorella* clade (including authentic strain *Leptochlorella corticola* CAUP H8401; HE984579), yet without statistical support. The isolates of *Chloroidium*, exhibiting widely elliptic cells with a band-shaped lobed chloroplast with a pyrenoid, were assigned to two species. (I) *C. saccharophilum* (e.g., isolate LH10HG7062; **Fig. 6h**), detected in grassland (HAI) and forest (ALB), is identical to authentic strain *C. saccharophilum* SAG 211-9a (FM946000) from tree sap. *Chloroidium* cf. *ellipsoideum* (e.g., isolate LH10HG9105; **Fig. 6i**), detected in grasslands (HAI, ALB), is closely related to authentic strain *C. ellipsoideum* SAG 3.95 (FM946012). The clade comprising both species is highly supported.

Coccoid isolates exhibiting predominantly spherical vegetative cells comprised phylogenetically divergent lineages *Lobosphaera* (Neustupa *et al.* 2011), *Xylochloris* (Neustupa *et al.* 2011), *Dictyochloropsis* (Dal Grande *et al.* 2014)*, Prasiola* clade (*'Chlorella' mirabilis*) and members of the Chlorellaceae (Krienitz *et al.* 2004). The isolates assigned to *Lobosphaera* exhibited spherical cells with one parietal chloroplast without a pyrenoid and could be phylogenetically classified in two species. (I) *Lobosphaera* cf. *irregularis* (isolates LH08SW5063, LH08AW3064; KP081398) from forests (ALB, SCH) is identical to *'Myrmecia' (= Lobosphaera) irregularis* CCAP 221/8 (HQ902935). (II) *Lobosphaera* cf. *bisecta* (isolate

LH10HG3P15; **Fig. 6j**) from grassland (HAI) is identical to authentic strain *Myrmecia (= Lobosphaera) bisecta* SAG 2043 (Z47209) from soil (Italy). The *Lobosphaera* clade (including as well *'Parietochloris' cohaerens* UTEX 1707; EU878372) is highly supported. *Xylochloris* sp. SAG 2382 (= LH08AG7024; **Fig. 6k**; JQ988942) from grassland (ALB), with characteristic parietal star-like lobed chloroplasts, is less closely related (similarity = 96.47%) to authentic strain *X. irregularis* CAUP H7801 (EU105209) from tree bark (Singapore). The clade including both *Xylochloris* accessions is only partly supported and the new *Xylochloris* sp. SAG 2382 is likely to represent new taxon on at least species or even genus level. Closely related genus *Dictyochloropsis* comprises *D. splendida* (isolate LH08AW3050; **Fig. 6l**; JQ988930) from forest (ALB), identical to *D. splendida* CAUP H8601 (GU017662) from soil near to a fumarole (Czech Republic). *Dictyochloropsis* and *Xylochloris* species built a well-supported clade. *Chlorella*-like isolates from the *Prasiola* clade, phylogenetically assigned to *'Chlorella' mirabilis*, were characterized by spherical cells with one parietal chloroplast containing characteristic small starch granules over the pyrenoid circumference. The isolates from grasslands in ALB (isolate LH08AG9040; **Fig. 7a**) and HAI (isolate LH10HG6139) are closely related to *'Chlorella' mirabilis* Andreyeva 748-I (X74000) from soil and might represent two different species. The clade including both isolates and *'Chlorella' mirabilis* Andreyeva 748-I is highly supported.

Chlorellaceae were represented by four different morphological types, i.e., *Chlorella*-like (spherical to slightly elliptic cells with cup-shaped chloroplast with one pyrenoid), *Auxenochlorella*-like (spherical cells with facultatively degraded chloroplasts; heterotrophic), *Muriella*-like (bigger spherical cells with single parietal chloroplast without a pyrenoid), *Nannochloris*-like (smaller spherical cells with single parietal chloroplast without a pyrenoid). *Chlorella vulgaris* isolates were sampled in grasslands (HAI, SCH) and forests (HAI). For example, *C. vulgaris* isolates LH08SG3006 and LH08HG1081 (**Fig. 7b**) were identical (similarity = 99.94-100%) to authentic strain of *C. vulgaris* SAG 211-11b (FM205832) from freshwater (Netherlands). *Chlorella* cf. *vulgaris* isolate LH10HG2081 (**Fig. 7c**) is closely related to *C. vulgaris* SAG 211-11b and identical to isolates L1 and L4 from soil (King George Island, Antarctica). The Antarctic strains (and isolate LH10HG2081) constitute a well-supported clade and might represent a new *Chlorella* species. *Muriella* cf. *terrestris* isolates LH08SG3009 and LH10HG7118 (**Fig. 7d**) from grasslands (HAI, SCH) are almost identical (similarity = 99.94%) to *Muriella terrestris* ASIB V38 (AB012845) from soil (Italy), within a highly supported clade. *Nannochloris*-like isolates clustered as four different species. (I) *Nannochloris* sp. isolates from grasslands (e.g., LH08SG8030, **Fig. 7e**; LH10HG6095, **Fig. 7f**) are almost identical to *N. bacillaris* (AB080300) within a highly supported clade. The remaining *Nannochloris*-like isolates

clustered within the highly supported Unidentified Chlorellaceae clade, a sister group to *N. bacillaris* (AB080300) and comprises three different (yet undescribed) species. One species comprises isolates from grasslands of HAI and ALB (e.g., isolate LH08AG1034; **Fig. 7g**), identical to *'Marvania'* sp. WB67 (KF144207) from Westerhöfer creek within a highly supported clade. Second species from grasslands (HAI, SCH) (e.g., isolate LH10HG9020; **Fig. 7h**) is identical to *Nannochloris* sp. JL-4-6 (AY195983) from freshwater (USA). Third species from grassland (SCH) (e.g., LH08SG3093; LH08SG3078, **Fig. 7i**) is identical to *Nannochloris* sp. Ant-1 (EF440182) from permafrost (Antarctica). *Auxenochlorella*-like colorless isolates represent two different taxa on either species or even genus level. (I) *A. protothecoides* (e.g., isolate LH10HG6096; **Fig. 7k**) from grasslands (HAI) is identical to authentic strain *A. protothecoides* SAG 211-7a (X56101) isolated from tree sap (Germany). This species clusters together with *Chlorella* sp. CCAP 211/61 (AB206551) in a highly supported clade. (I) *Auxenochlorella* sp. SAG 2478 (= LH08AW4103; **Fig. 7l**; KP081390) from forest (ALB), is less closely (similarity = 97.46%) related to *A. protothecoides* SAG 211-7a, without any known closer relatives, probably representing either new species or genus. The whole clade including *Auxenochlorella* sp. and *A. protothecoides* is highly supported. Another highly supported clade, consisting of isolate LH10HG709K (**Fig. 7j**) from grassland soil (HAI) and clone HEW1B K3342 from forest soil (HAI), does not include other known relatives (not shown in phylogenetic trees in this Chapter, but in **Fig. 2 Chapter 3**). The species might represent a new genus with basal placement among the *Nannochloris*-like lineages within the Chlorellaceae.

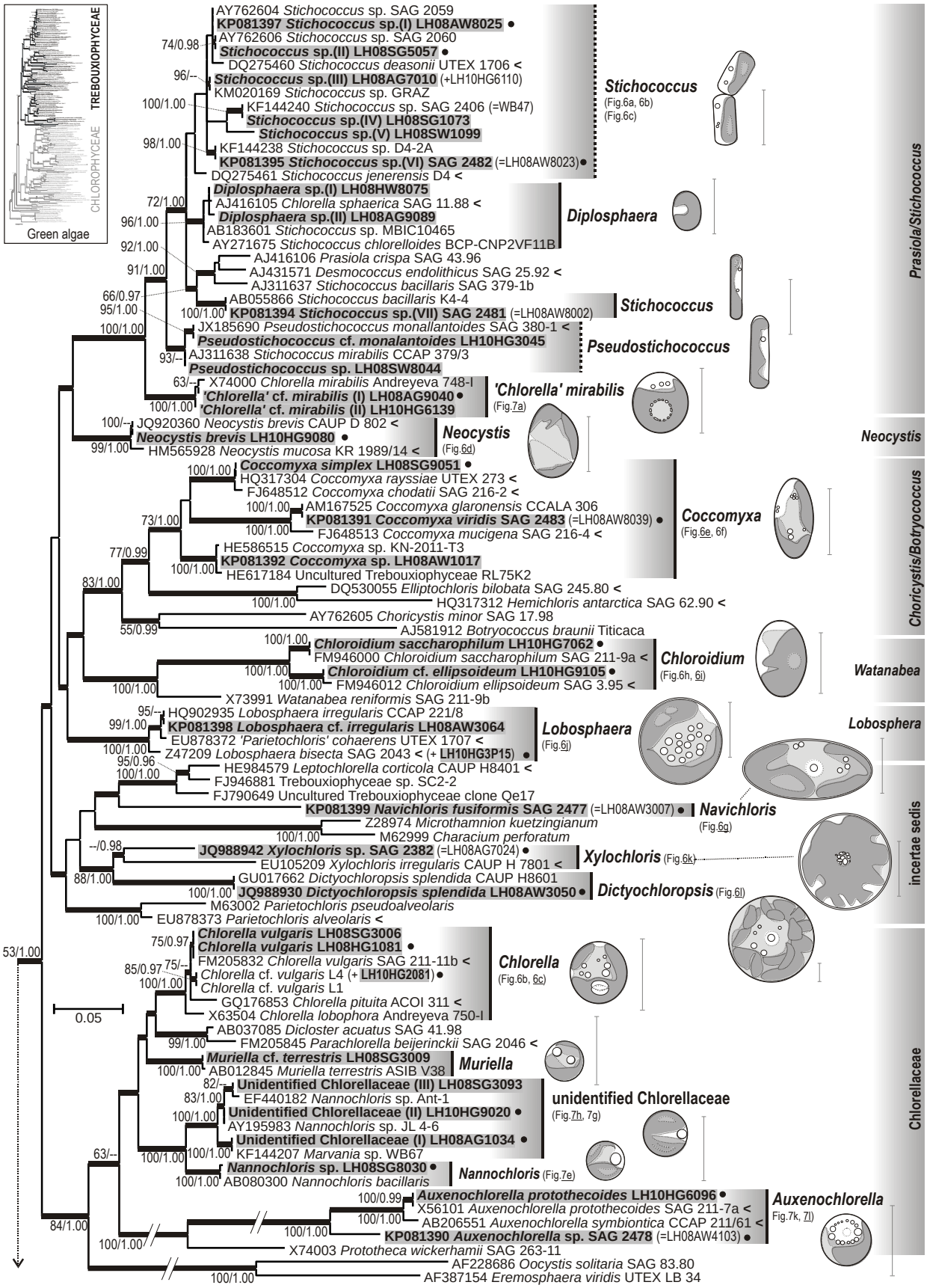

Figure 5. 18S rDNA phylogeny of Green algae, part 2 including Trebouxiophyceae. New accessions are written in bold and grey underlaid. Black dots mark references to microphotographs (**Fig. 6**, **Fig. 7**) sorted in top down order. Original drawings of representative morphologies are shown at each clade (scale bars = 5µm). Sequences of authentic strains are marked by '>'. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities).

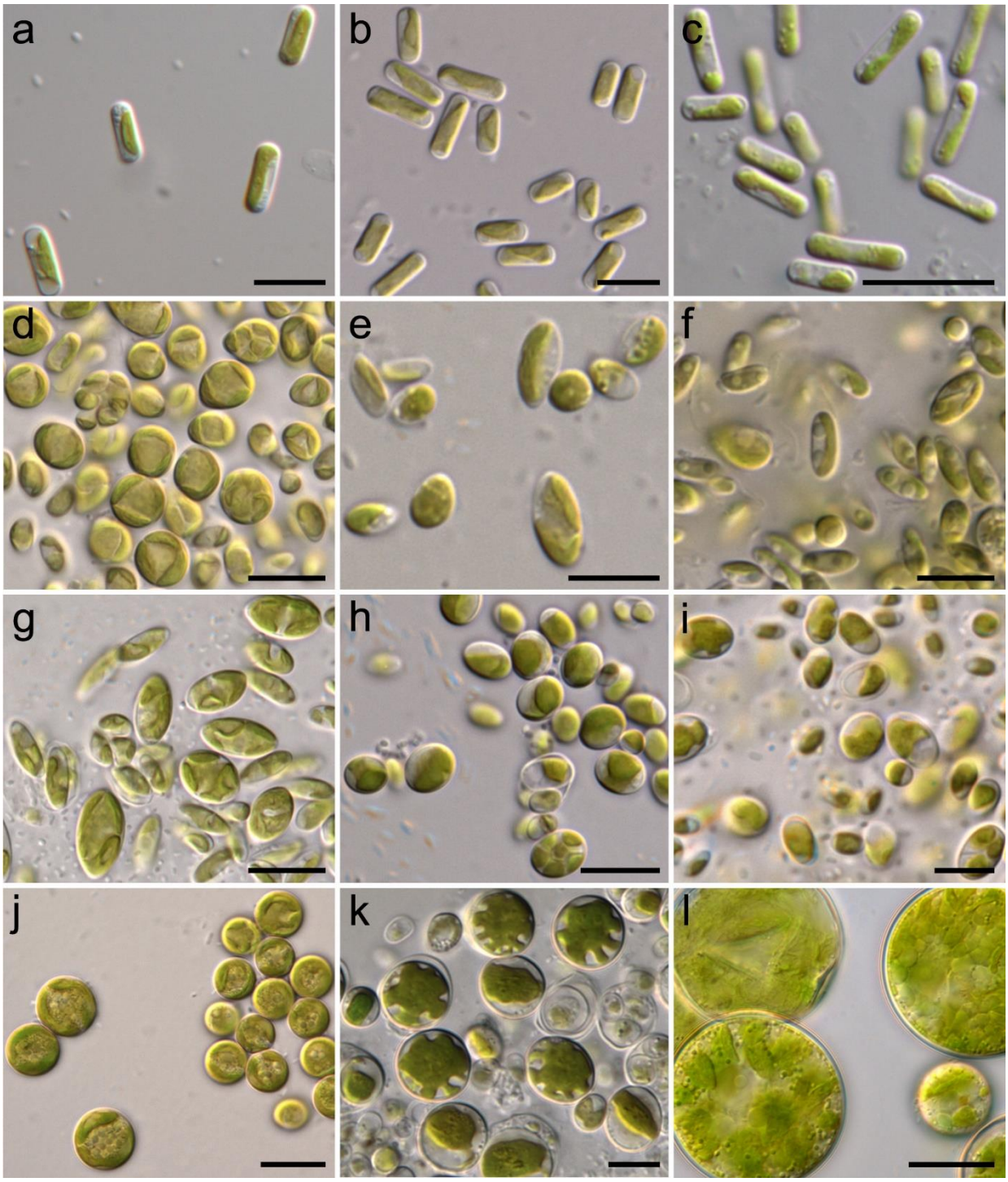

Figure 6. Microphotographs of monoclonal cultures of Green algae (Trebouxiophyceae). (a) *Stichococcus* sp.(I) LH08AW8025 (l=1.7-19.8 µm, w=1.4-3.6 µm); (b) *Stichococcus* sp.(II) LH08SG5057 (l=4.5-9.8 µm, w=1.9-3.3 µm); (c) *Stichococcus* sp.(VI) SAG 2482 (= LH08AW8023; l=3.8-7.2 µm, w=1.9-6.3 µm); (d) *Neocystis brevis* LH10HG9080 (l=4.8-8.7 µm, w=2.7-6.6 µm); (e) *Coccomyxa simplex* LH08SG9051 (l=4.4-7.8 µm, w=2.0-4.3 µm); (f) *Coccomyxa viridis* SAG 2483 (= LH08AW8039; l=4.7-8.4 µm, w=1.8-3.6 µm); (g) *Navichloris fusiformis* SAG 2477 (= LH08AW3007; l=8.3-15.3 µm, w=3.6-7.8 µm); (h) *Chloroidium saccharophilum* LH10HG7062 (l=5.6-9.1 µm, w=4.0-6.2 µm); (i) *Chloroidium* cf. *ellipsoideum* LH10HG9105 (l=4.8-7.8 µm, w=3.3-5.3 µm); (j) *Lobosphaera bisecta* LH10HG3P15 (Ø=6.2-9.6 µm); (k) *Xylochloris* sp. SAG 2382 (= LH08AG7024; l=4.7-12.9 µm, w=3.1-10.6 µm); (l) *Dictyochloropsis splendida* LH08AW3050 (Ø=7.7-39.4 µm). Scale bars = 10 µm.

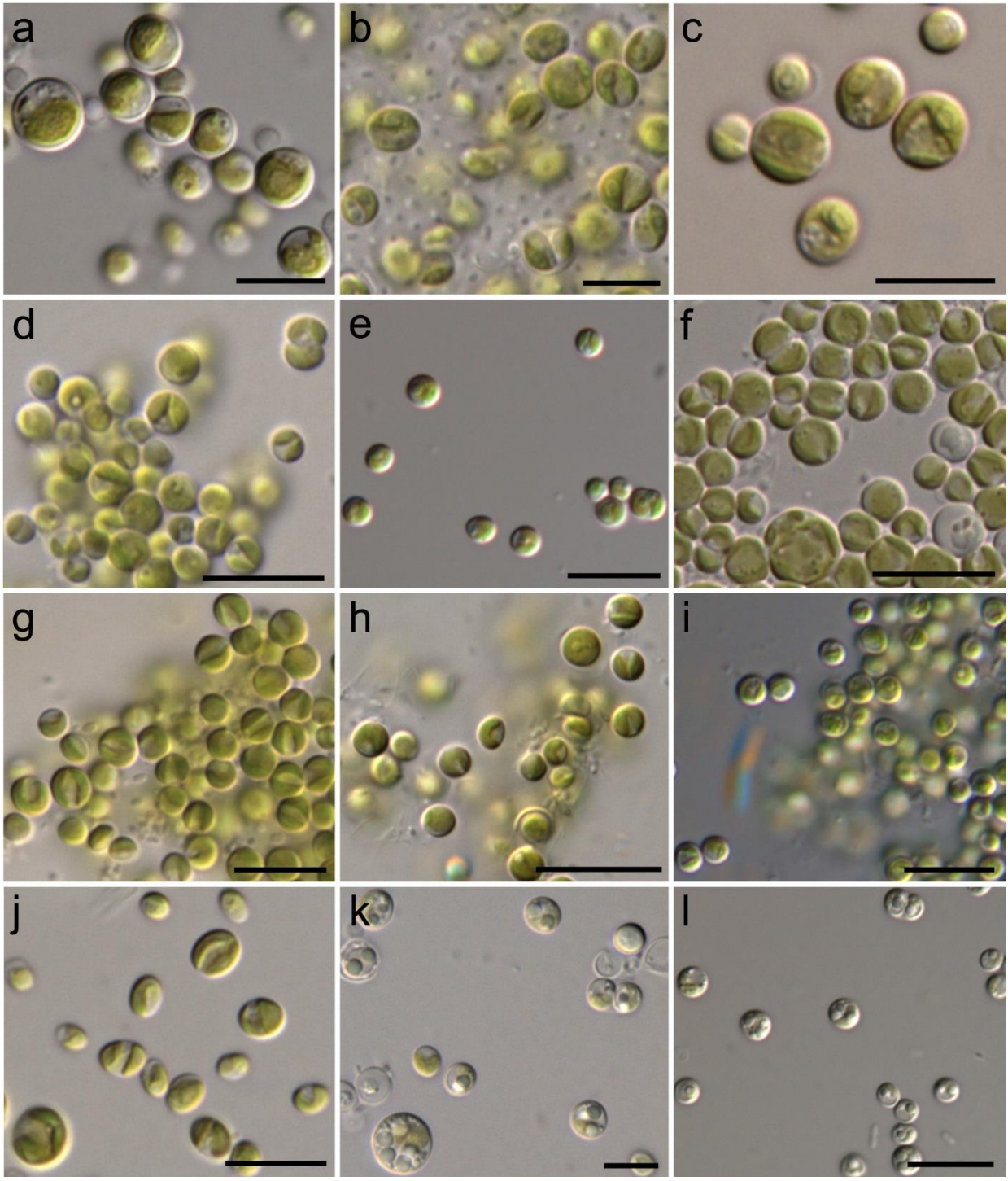

Figure 7. Microphotographs of monoclonal cultures of Green algae (Trebouxiophyceae). (a) *'Chlorella'* cf. *mirabilis* LH08AG9040 (Ø=3.0-6.4 µm); (c) *Chlorella* cf. *vulgaris* LH10HG2081 (Ø=2.5-6.2 µm); (d) *Muriella terrestris* LH10HG7118; (e) *Nannochloris* sp. LH08SG8030 (Ø=2.2-3.9 µm); (f) *Nannochloris* sp. LH10HG6095 (Ø=2.1-3.9 µm); (g) Unidentified Chlorellaceae (I) LH08AG1034 (Ø=2.4-4.5 µm); (h) Unidentified Chlorellaceae (II) LH10HG9020 (Ø=2.4-4.8 µm); (i) Unidentified Chlorellaceae (III) LH08SG3078 (Ø=2.1-3.7 µm); (j) Unidentified Chlorellaceae LH10HG709K (l=3.1-5.1 µm, w=2.0-3.7 µm); (k) *Auxenochlorella protothecoides* LH10HG6096 (Ø=2.9-6.5 µm); (l) *Auxenochlorella* sp. SAG 2478 (= LH08AW4103; Ø=2.2-5.2 µm). Scale bars = 10 µm.

Geographic dispersal and taxonomic 'novelty' of the isolated Green algae

In total, we identified 61 species of Green algae. Thirty-nine (53%) species were detected once and 34 (47%) species were detected more than once (e.g., *Chlorella vulgaris*; 19 times; **Table S4a**). Thirty (41%) species were retrieved from more than one plot and 18 (25%) species were retrieved from more than one exploratory (**Fig. 8**; **Table S4b**). There were only two species which we detected in all three exploratories, i.e., *Stichococcus* sp. (II) and *Neocystis brevis* (**Table S4b**). Almost all clades included both forest and grassland species, with the only exception of the Chlorellaceae with most species detected only in grasslands (**Fig. 8**). More than a half of all detected species (61%) exhibited > 99.90% sequence similarity to accessions known from previous studies. But only less than a half of these accessions belong to validly named (authentic) strains (**Fig. 9a**). Many next relatives to our species originate from Europe (54%), most of them from soil (30%) or various aquatic habitats (27%). Species less closely related to allready published accessions (< 99.98% sequence similarity) mostly originate from grassland plots (62%; **Fig. 9a**). ITS2 sequence comparisons revealed occurrences of identical ribotypes separated by considerable distances, e.g., *'Chlorella' mirabilis*, *Chlorella vulgaris*, *Pseudostichococcus monallantoides* and *Klebsormidium dissectum* (**Fig. 9b**). Other notably high similarities were detected in *Chlamydopodium vaculolatum* (99%) and *Muriella terrestris* (98%) (**Fig. 9b**).

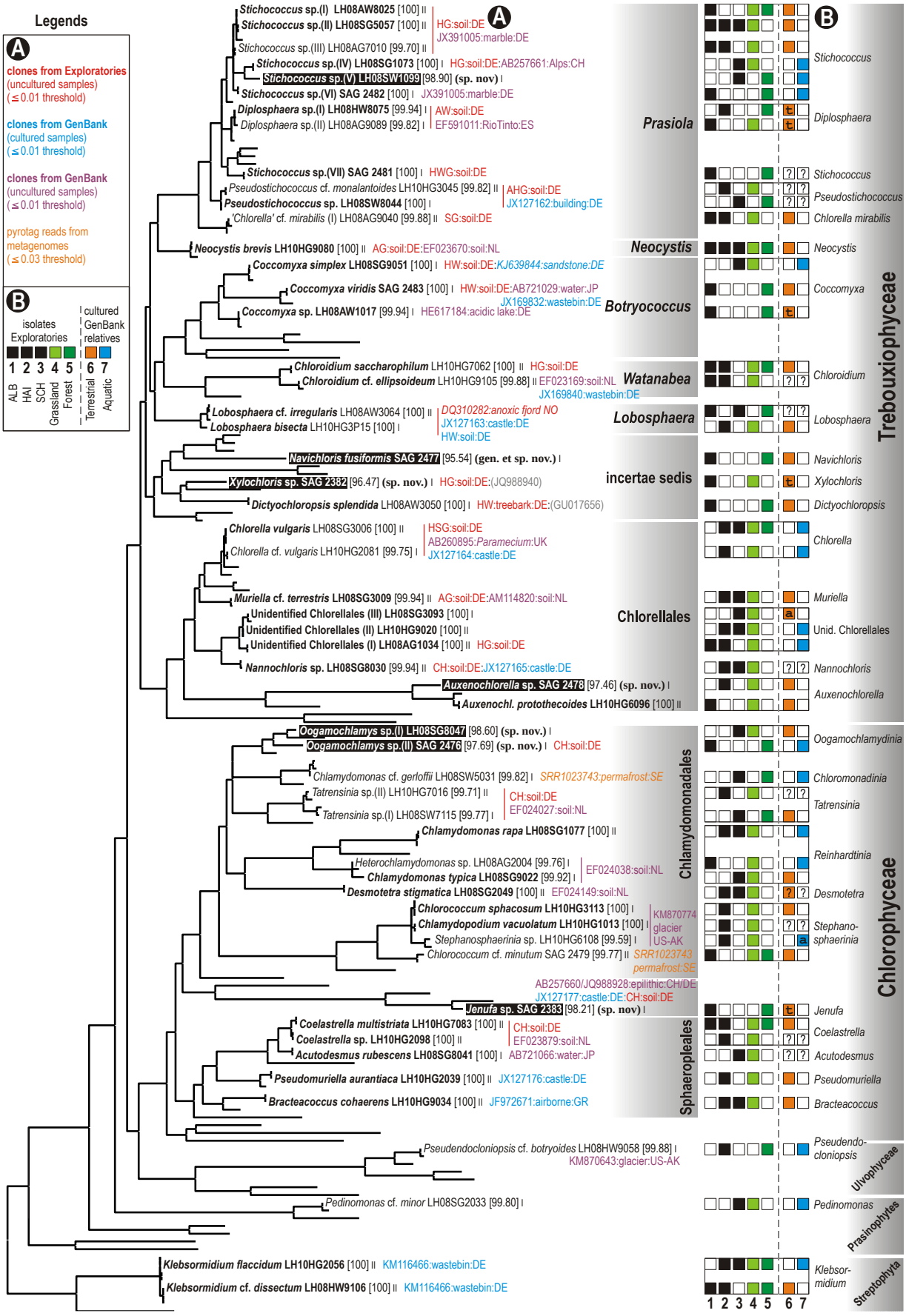

Figure 8. Diversity and distribution of all green microalgae isolated within this study. See further explanations on next page.

Figure 8. Diversity and distribution of all Green microalgae isolated within this study. The 18S phylogenetic tree gives an overview of all detected species (each species is represented by one sequence in the tree; due to a lack of space, names of reference sequences were removed from the figure; the comprehensive taxon samplings are shown in **Fig. 2**, **Fig. 5**). Numbers in brackets give percentage sequence similarity to the closest relatives. 'I' or 'II' indicate whether a species was found only once or multiple times within the Exploratories. Colored squares to the right of the tree summarize the distribution of each species across the three Exploratories (ALB, HAI, SCH) and across the two different habitats (grassland, forest). This evidence is based on sequenced isolates listed in **Table S4**. Further two squares indicate whether the closest GenBank-relative originate from terrestrial or aquatic habitats. Numbers in brackets behind isolate identifiers indicate (18S-based) sequence similarity to the closest cultured relatives. Algal clones, which are closely related to our isolates, are differentiated by colors due to their source (red: environmental clones obtained from the same sampling sites (Hallmann *et al.* in prep.-b); purple: GenBank accessions from culture-independent surveys; blue: GenBank accessions from culture-dependent studies).

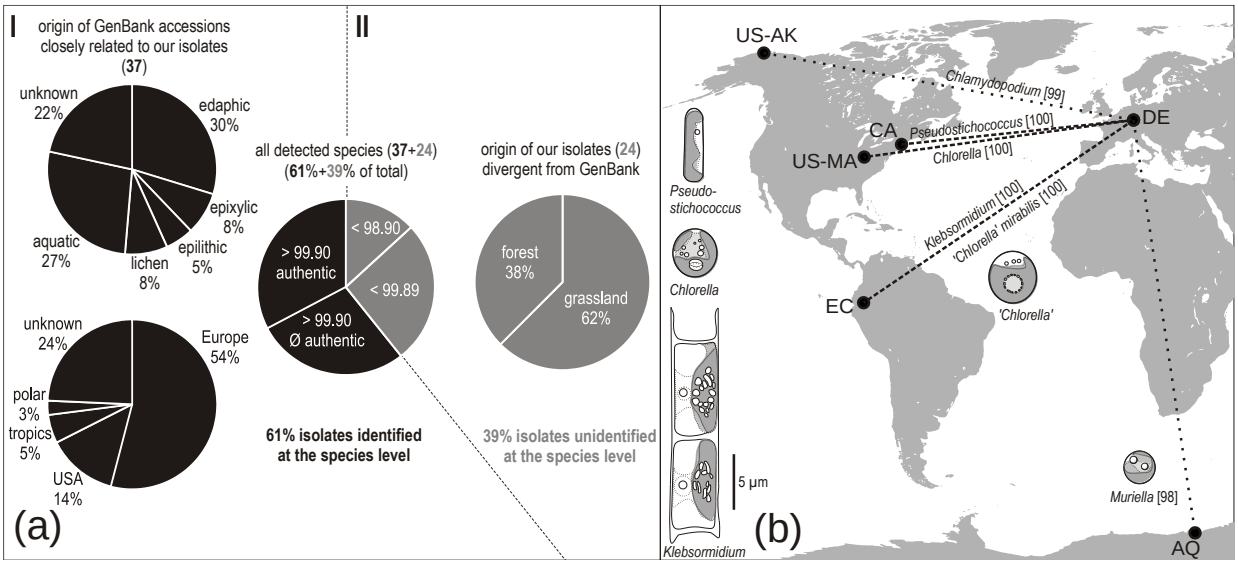

Figure 9. Geographic distribution of Green algal species isolated from soils. (a) Pie charts derived from 18S rDNA sequence similarities illustrate (I) how many isolates could be identified at species level (and where their closest relatives originated from), (II) how many isolates represent undescribed species (and from which habitats these undescribed species originated from); (b) Map showing places where identical or highly similar ITS2 rDNA sequences were found. Evidence for 100%-identity: *Stichococcus* LH08SW8044 (DE) and SAG 379-4 (CA), *Chlorella* LH08SG3006 (DE) and FN298918 (US-MA), *Klebsormidium* LH08AG1113 (DE) and KS164CL6L (EC), *'Chlorella' mirabilis* LH08AG9040 (DE) and KFFB12-1 (EC); Evidence for high similarity [98-99%]: *Chlamydopodium* LH10HG1013 (DE) and FR865591 (US-AK), *Muriella* LH08SG3009 (DE) and JN653521 (AQ). CA=Canada, DE=Germany, US-AK=Alaska, US-MA= Massachusetts, EC=Ecuador. AQ=Antarctica.

Stramenopiles: Xanthophyceae

Phylogenetic analysis of the Stramenopiles (**Fig. 10a**), assigned isolates to the classes Xanthophyceae (six clades) and Eustigmatophyceae (one clade) (**Fig. 10b**).

Tribonematalean lineage (Maistro *et al.* 2009). Tribonematalean isolates with unbranched filaments could be assigned to *Xanthonema* (three species) and *Heterothrix* (two species). (I) *Xanthonema* sp. (e.g., isolates LH10HG7029, LH10HG9058; **Fig. 11a**), detected in grassland (HAI) and forest (HAI), is closely related to *X. exile* PAB 395 (AM491615). (I) *Xanthonema* cf. *exile* (isolate LH10HG7078; **Fig. 11b**) from grassland (HAI) is almost identical to *X. exile* PAB 395 (AM491615). (III) *Xanthonema* cf. *bristolianum* (isolates LH10HG6059, LH08HW9018;

Fig. 11c) from grassland (HAI) and forest (HAI), is identical to terrestrial *X. bristolianum* CCALA 516 (AM490819) isolated from snow (Slovakia). The *Xanthonema* clade is well supported. (IV) *Heterothrix* sp. (isolates LH10HG7061, **Fig. 11d**; LH08SG5052, **Fig. 11e**) from grasslands (HAI, SCH) is related to *Heterothrix* sp. ACOL A1 (AM491612) and might represent a new or undescribed species. (V) *H. sessile* (e.g., isolate LH10HG5079) from grassland (HAI) is identical to *H. sessile* IBSG-V28 (AM490818). The *Heterothrix* clade is highly supported.

Chlorellidialean lineage (Maistro *et al.* 2009). Chlorellidialean heterotrichal isolates clustered in three different species of *Heterococcus*. (I) *Heterococcus* sp. (isolates LH10HG2140, LH10HG9085; **Fig. 11f**) from grasslands (HAI) is related to freshwater Xanthophyceae sp. IX3 (FJ946906) from Antarctica and might represent a new species. (II) *Heterococcus* cf. *chodatii* (e.g., isolate LH10HG9111; **Fig. 11g, 11h**) from grasslands (HAI) is identical to authentic strain *H. chodatii* SAG 835-3 (AM490822) from a subaerial habitat (Switzerland). The clade including *H. chodatii* (and *H. pleurococcoides* PAB 380; AJ579335) is well supported. (III) *Heterococcus* cf. *caespitosus* (isolate LH08AG2020) from grassland (ALB) is almost identical to authentic strain *H. caespitosus* SAG 835-2a (AM490820) from soil (Germany) and at the same to *H. protonematoides* SAG 835-9 (AJ579334) from soil (Switzerland). The clade including both species is highly supported, as well as the whole *Heterococcus* clade.

Botrydiopsalean lineage (Maistro *et al.* 2009). Botrydiopsalean isolates (two species) exhibited either cell packages or solitary spherical cells with numerous discoidal chloroplasts. (I) Botrydiopsalean sp. (e.g., isolate LH08AW1076; **Fig. 11i**) from grassland (SCH) and forest (ALB) is related to *Chlorellidium pyrenoidosum* PAB 785 (AJ579338) from soil (Antarctica). The clade including both species is not supported and the isolate LH08AW1076 might represent a new species. (II) *Botrydiopsis* sp. (isolate LH08AW4043; **Fig. 11j**) from forest (ALB) is related to *B. callosa* SAG 30.83 (AJ579340) from soil (Italy). The clade including both species is well supported and the isolate LH08AW4043 might represent a new species.

The thallous genus *Asterosiphon* Dangeard is represented by *Asterosiphon* sp. (isolate LH10HG3064; **Fig. 11k**) from grassland (HAI), which is less closely related (similarity = 98.54%) to *A. dichotomus* UTEX LB 2066 (AM490829). *Asterosiphon* sp. LH10HG3064 exhibited filamentous and coccal stages in the culture. The *Asterosiphon* clade is well supported and the isolate LH10HG3064 probably represents a new species.

Stramenopiles: Eustigmatophyceae

The genus *Eustigmatos* Hibberd is represented by *Eustigmatos* sp. (isolates LH10HG5036, LH10HG9133; **Fig. 11l**) from grasslands (HAI) is closely related to *E. magna* CCMP 387

(U41051) from soil (New Zealand). Both isolates exhibit sphaerical cells with characteristic angular shaped 'false pyrenoid'. The clade including both isolates and *E. magna* CCMP 387 is supported.

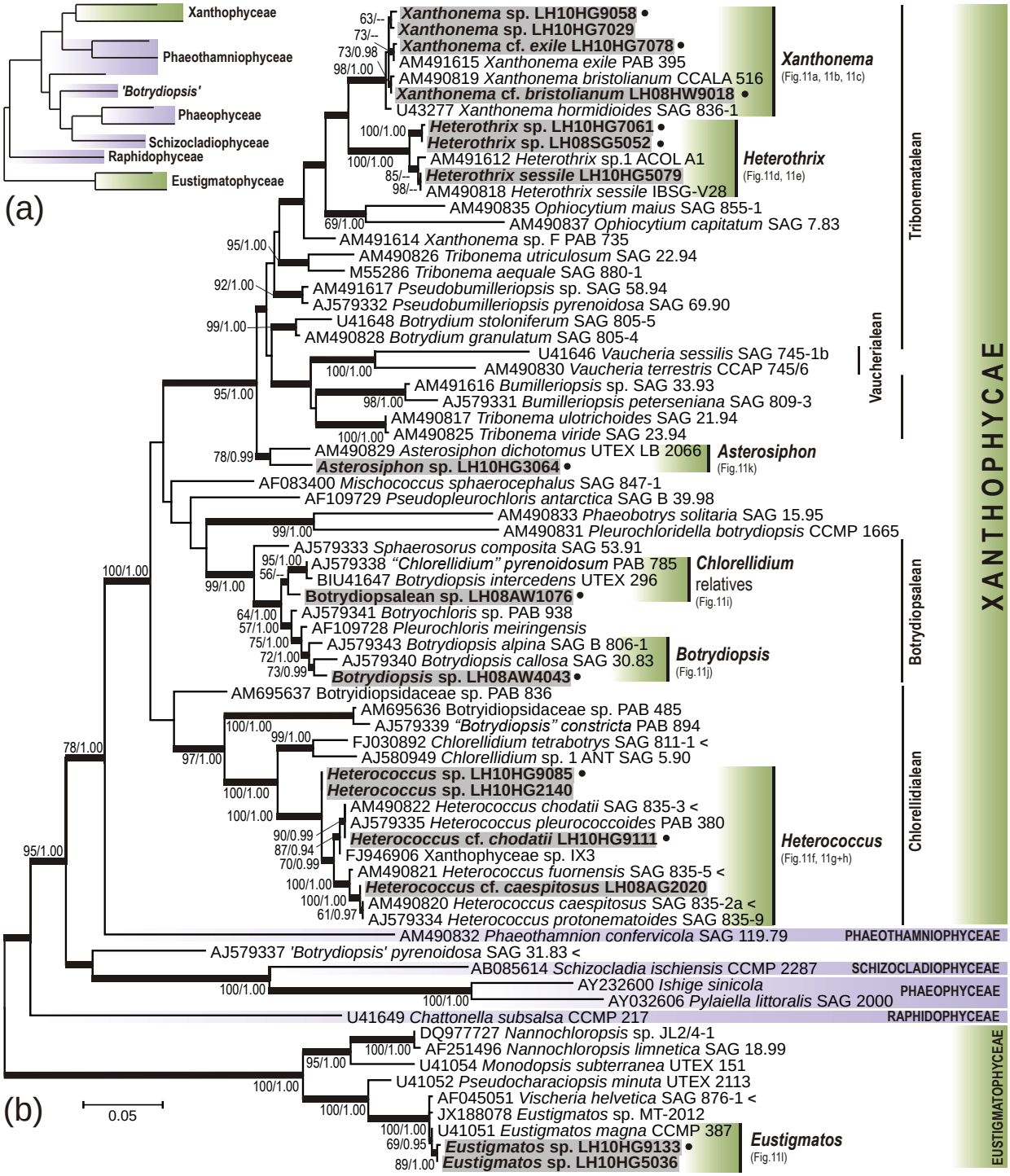

Figure 10. Phylogeny of some Stramenopiles, including Xanthophyceae and Eustigmatophyceae. (a) Schematic multi-gene phylogeny (modified after Maistro *et al.* (2009)) of the same Stramenopile groups which were used for the 18S phylogeny. (b) 18S rDNA phylogenetic tree showing placement of the new isolates (grey underlaid; black dots mark isolates which are shown on microphotographs (**Fig. 11**). References to the microphotographs (**Fig. 11**, listed below clade names) correspond to black dots in top down order. Sequenced authentic strains are marked by a '<' sign. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities).

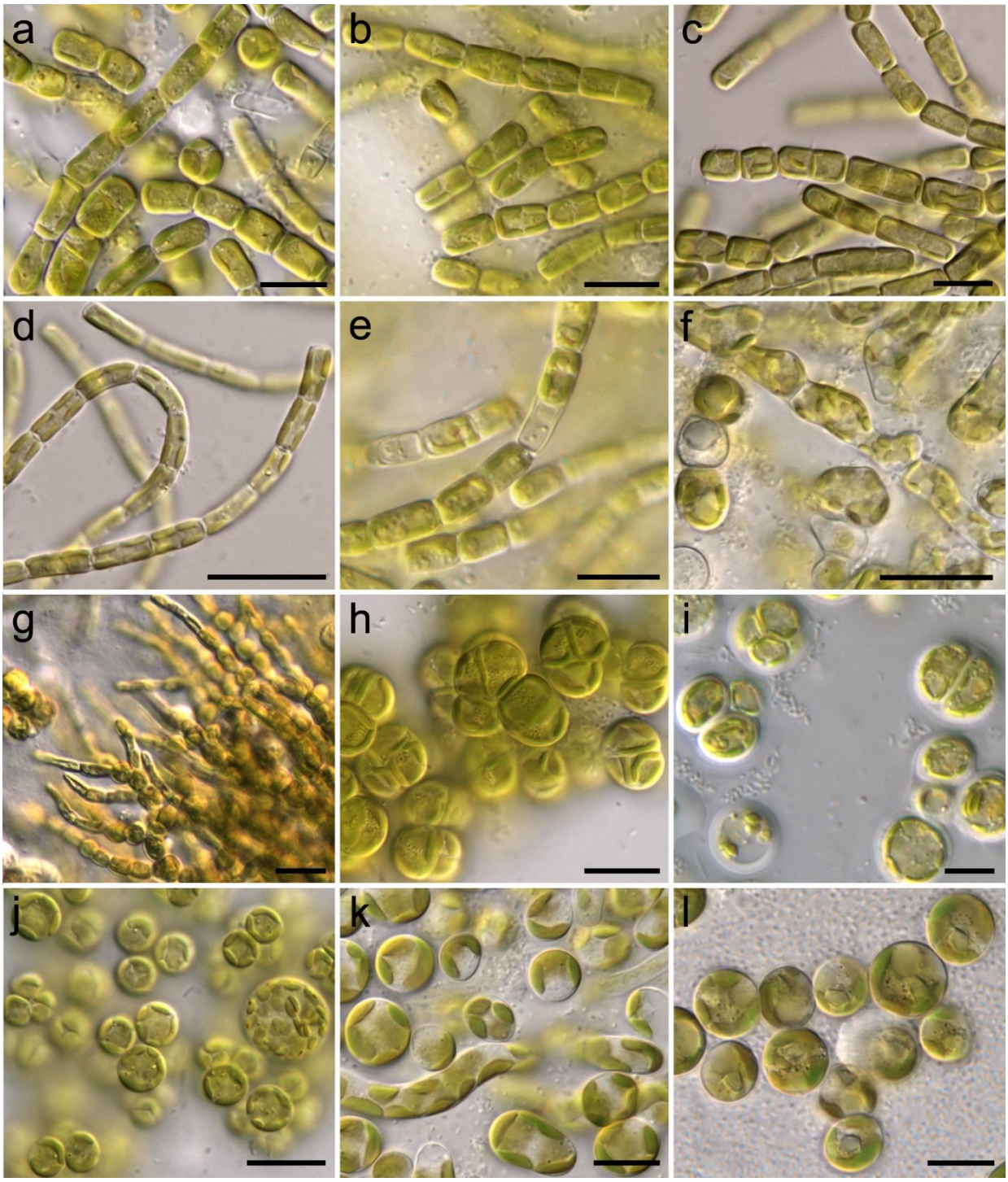

Figure 11. Microphotographs of monoclonal cultures (Trebouxiophyceae, Xanthophyceae, Eustigmatophyceae). (a) *Xanthonema* sp. LH10HG9058; (b) *Xanthonema exile* LH10HG7078; (c) *Xanthonema* cf. *bristolianum* LH08HW9018; (d) *Heterothrix* sp. LH10HG7061; (e) *Heterothrix* sp. LH08SG5052; (f) *Heterococcus* sp. LH10HG9085; (g) *Heterococcus* cf. *chodatii* LH10HG9111; (h) *Heterococcus* cf. *chodatii* LH10HG9111; (i) Botrydiopsalean sp. LH08AW1076; (j) *Botrydiopsis* cf. *callosa* LH08AW4043; (k) *Asterosiphon* sp. LH10HG3064; (l) *Eustigmatos* sp. LH10HG9133. Scale bars = 10 µm.

Morphological diversity observed in cultured soil samples

Microscopic investigation of the cultured soil samples (liquid cultures, agar plates, cover slips; **Fig. 1g-i**) revealed 45 distinct morphotypes (**Figs. S1, S2, S3, S4**). We compared the diversities among the Exploratories and between the two soil horizons (**Fig. S5a-b**). The A-horizon counts lower morphotype diversity (22 morphotypes; 57 plots/57 samples) than the O-horizon (46 morphotypes; 5 plots/20samples; **Fig. S5b**). The highest diversity exhibited Chlorophyta/Streptophyta (24), followed by Bacillariophyceae (8), Cyanobacteria (7), Xanthophyceae/Eustigmatophyceae (5), Rhodophyta (1) and Cryptophyta (1) (**Table S6, S7**). In general, the number of morphotypes is nearly the same in grasslands and forests (**Fig. S5b-c**), irrespective of the sampling technique (drill cores, soil surface samples). The A-horizon sampling revealed negligible differences in alpha-diversity between the three regions. In average, intermediately managed plots were slightly more diverse than extensively and intensively managed plots (**Fig. S5f**). The same applies for forests and grasslands, yet without statistical support. However, the morphotype composition differed between forest and grassland plots, explained through differences in physico-chemical parameters (**Fig. S5d**). The statistically significant ordination model ($P < 0.05$) based on Redundancy Analysis (RDA) revealed separation of forest and grassland replicate samples along the first ordination axis which is correlated with pH values ($R = 0.74$; $P < 0.05$). The contribution of total nitrogen ($R = 0.26$; $P < 0.05$) and organic carbon ($R = 0.11$; $P < 0.05$) is lower. The difference between soil communities in forests and grasslands can be explained by higher diversity of Cyanobacteria, Diatoms and Xanthophytes in grasslands. Considering the O-horizon (soil surface samples from HAI), multiple samples within a single plot exhibited different morphotype compositions (**Fig. S5e**). The ordination based on NMDS analysis (Shepard stress = 0.11) separates forest and grassland samples (axis 1) and points out higher heterogeneity of the forest plots (axis 2).

Discussion

Culturing of soil samples from 30 forest and 27 grassland sites retrieved 188 monoclonal cultures of Green microalgae. We inferred conspecifity of the sequenced isolates based on ≤ 0.001 SSU dissimilarity threshold. In multiple cases, our isolates were divergent from already sequenced authentic strains available from GenBank. Therefore, to denominate detected species, we relied on the phylogenetic species concept (Leliaert *et al.* 2009; Leliaert *et al.* 2014), not strictly following the taxonomical nomenclature (Cantino and de Queiroz 2007; McNeill *et al.* 2012). This approach distinguished 61 species covering five classes of Green algae: Chloro-,

Klebsormidio-, Pedino-, Trebouxio- and Ulvophyceae. In the following we focus mainly on two classes accounting for the most detected species - Trebouxiophyceae (36 spp.; 8 lineages) and Chlorophyceae (21 spp.; 10 lineages). We discuss whether our soil isolates can be recognized in already denominated algal strains deposited in public culture collections (e.g., SAG, UTEX, CCAP). Then we trace the distribution of our species by analysing records in GenBank and discuss their putative detectability in different environments by culture-independent sequencing.

New soil isolates and their cultured relatives

More than 50% of Green microalgae we isolated from soils are putatively conspecific with isolates from previous studies (**Fig. 8**; **Fig. 9a**). The most of these conspecific cultures originate from Central and Western Europe (54%) and from the USA (14%), corresponding to strains deposited in the Culture Collection of Algae in Göttingen (SAG) incl. their derivatives in UTEX or CCAP. Exotic counterpart cultures were few, e.g., *Coccomyxa* sp. (HE586515) from Indonesia or *Nannochloris* sp. from Antarctica (EF440182; Gilichinsky *et al.* 2007). Sequenced strains—conspecific with our isolates—originate from terrestrial (edaphic, epilithic, epixylic/lichenized) and aquatic environments. So far, there are no estimates of total diversity of microscopic Green algae in soils. However, the number of cultured species which match environmental clones, suggests that the uncultured fraction is not as extensive as in bacteria (99%; Hirsch *et al.* 2010). All 61 isolated species grow easily on agarized (solid) media, which are selective and might favor so called 'weedy' or generalists species (Škaloud *et al.* 2014a). Additional enrichment techniques (growth in liquid media or on coverslips), indeed, retrieved additional species, which we did not retrieved as monoclonal cultures for sequencing (e.g., *Macrochloris*, *Spongiochloris*, *Keratococcus*, *Podohedra*, *Scotinosphaera*, *Pseudendoclonium*, *Interfilum* or *Cosmarium*; **Fig. S1**, **S2**). The identification literature by Ettl and Gärtner (1995) covers about 500 morphospecies of terrestrial Green microalgae—partially documented as sequenced strains deposited in culture collections. We re-detect some of them in the present study (via SSU-comparisons) and could either support or reject their ecological preference (so far inferred via morphospecies-based approaches). Among such sequenced Green microalgae, which were documented by particular algal strains in (Ettl and Gärtner 1995), we confirmed characteristic grassland species: *Bracteacoccus cohaerens* UTEX 1272 (Bischoff and Bold 1963), *'Chlorococcum' gelatinosum* SAG 64.80 (Archibald and Bold 1970), *Chlamydomonas typica* NIES-2246 (Deason and Bold 1960), *'Chlorella' mirabilis* Andreyeva 748-I (Andreyeva 1973), *Desmotetra stigmatica* UTEX B 962 (Deason and Floyd 1987), *Muriella terrestris* ASIB V38 (Ettl and Gärtner 1995) and *Pseudomuriella aurantiaca* SAG 249-1 (Hanagata 1998). A few species were repeatedly verified

in forests, e.g., *Chloromonas gerloffii* and *C. rosae* (Ettl 1963) or in soils of both forests and grasslands, e.g., *Lobosphaera bisecta* SAG 2043 (Trenkwalder 1975), *Chlorococcum minutum* ASIB T50 (Trenkwalder 1975) and *Heterochlamydomonas inaequalis* UTEX 1705 (Cox and Deason 1969). Finally, in our soil samples we recognized well documented terrestrial generalists such as: *Chloroidium saccharophilum* SAG 211-9a (Darienko *et al.* 2010), *Dictyochloropsis splendida* CAUP H8601 (Škaloud *et al.* 2005) and *'Chlorella' sphaerica* SAG 11.88 (Tschermak-Woess 1988). Despite the sampling efforts in forests, we did not retrieve species characteristic for temperate tree-bark biofilms or lichens, e.g., *Apatococcus* (Hallmann *et al.* 2009; Hallmann *et al.* in prep.-a), *Elliptochloris* (Eliáš *et al.* 2008), *Kalinella* (Neustupa *et al.* 2009), *Leptochlorella* (Neustupa *et al.* 2013a), *Parachloroidium* (Neustupa *et al.* 2013b), *Trebouxia* (Friedl and Rokitta 1997; Kroken and Taylor 2000) or *Asterochloris* (Škaloud *et al.* 2015). This might be due to selectivity of the used culturing medium, reflecting nutrient demands of soil but not corticolous species.

New taxa detected in soils

Seven isolates are considerably divergent from all GenBank clones or cultures, presumably representing novel taxa. The novel Chlorophyceae are: *Jenufa* sp. SAG 2383, *Oogamochlamys* sp.(I) LH08SG8047, *Oogamochlamys* sp.(II) SAG 2476. The novel Trebouxiophyceae are: *Auxenochlorella* sp. SAG 2478, *Stichococcus* sp.(V) LH08SW1099, *'Navichloris fusiformis'* SAG 2477, *Xylochloris* sp. SAG 2382. *Jenufa* Němcová, Eliáš, Škaloud & Neustupa represents a coccoid genus and at the same time a lineage incertae sedis within the class Chlorophyceae. Two terrestrial species were described from Southeast Asia (Němcová *et al.* 2011) and multiple congeneric accessions were acquired also from Neotropics (Hodač *et al.* 2012), the Alps (Horath and Bachofen 2009) and finally in the frame of this study (*Jenufa* sp. SAG 2383). The only European isolate *Jenufa* sp. SAG 2383 (**Fig. 4l**) might represent commonly occurring terrestrial species detected in forest soils and epilithic biofilms (Hodač *et al.* 2012).

Oogamochlamys Pröschold, Marin, Schlösser & Melkonian is a monadoid genus within the *Oogamochlamydinia* clade (Chlamydomonadales). The three species described by Pröschold *et al.* (2001) genetically differ from our two congeneric isolates provisionally denominated *Oogamochlamys* sp.(I) LH08SG8047 **(Fig. 4a)** and *O.* sp.(II) SAG 2476 **(Fig. 4b)**. Distribution and ecology of both species remains unclear, as they were detected only once, *Oogamochlamys* sp.(I) in one forest site and *O.* sp.(II) in one grassland site. Remarkably, all other described *Oogamochlamys* species were isolated from soils in Africa and North America (Pröschold *et al.* 2001). And at least one another verifiably congeneric isolate—*Chlamydomonas* sp. CCAP

11/159—was detected in a lake in North America (GenBank acc. no. FR865591).

Auxenochlorella (Shihira & Krauss) Kalina & Punčochářová is a genus within the Chlorellaceae comprising three facultatively heterotrophic species (Darienko and Pröschold 2015; Rodó and Molinari-Novoa 2015). The new facultatively heterotrophic isolate *Auxenochlorella* sp. SAG 2478 (**Fig. 7l**) is verifiably congeneric with all described *Auxenochlorella* species but is still considerably divergent from all accessions available in GenBank. *Auxenochlorella* sp. SAG 2478 is the first species of this genus which was isolated from soil. Other *Auxenochlorella* species were isolated from different terrestrial and aquatic habitats, e.g., tree sap and *Hydra viridis* endosymbiont (Darienko and Pröschold 2015).

Stichococcus Nägeli is a common denomination of rod-shaped Green algae from the *Prasiola* clade, counting 50 described species (Karbovska and Kostikov 2012a). Only two *Stichococcus* species were verified by genetic data and the polyphyletic taxon awaits taxonomical revision. All seven *Stichococcus*-like species detected in the frame of this study represent new taxa, however, six of them are closely related with already existing cultures or clones. Except for the isolate *Stichococcus* sp. LH08SW1099 (**Fig. 8**; **Fig. 5i Chapter 3**).

Xylochloris Neustupa, Eliáš & Škaloud is a genus incertae sedis within the Trebouxiophyceae with one described terrestrial species *X. irregularis* isolated in Southeast Asia (Neustupa *et al.* 2011). Our soil isolate *Xylochloris* sp. SAG 2382 (**Fig. 6k)** is putatively congeneric with *X. irregularis*, together with clones from European soils and tree-barks in South America (Hodač *et al.* 2012; Spitzer *et al.* 2014). The genus is probably widely distributed in Europe, as recently reported from tree-bark in the Southern Europe (Kulichová *et al.* 2014).

Navichloris fusiformis L.Hodač et al., ad interim (**Fig. 6g**) is a new species and genus of an undescribed lineage within the class Trebouxiophyceae. The existence of this novel lineage was already proposed by Lewis and Lewis (2005), who phylogenetically analyzed the strain *Chlorella* sp. BC4VF9 from Baja California, Mexico, without affiliation to any known trebouxiophycean clade. Costello *et al.* (2009) pointed out close relationship of *Chlorella* sp. BC4VF9 and two clones sampled from cold-fumarole soil on the Socompa volcano, Atacama, Chile. However, the obviously novel trebouxiophycean clade remained undescribed, since the only existing culture *Chlorella* sp. BC4VF9 died (Fučíková *et al.* 2014). Phylogenetic analysis (**Fig. 12**) show that the three desert accessions cluster with the strain SAG 2477 isolated in the frame of this study from a forest soil in Schwäbische Alb, Germany. The clade is statistically supported and represents a phylogenetically unique lineage of terrestrial trebouxiophycean microalgae. The strain SAG 2477 as well exhibits a unique fusiform/elliptic morphology, generally resembling *Coccomyxa* (Schmidle 1901). However, phylogenetically it is not related to any member of the recently

revisited genus *Coccomyxa* (Darienko *et al.* 2015). The characteristic feature of the strain SAG 2477 are lens-like light zones in the chloroplast lobes (**Fig. 6g**), similar to *Elliptochloris incisiformis* Hoffmann & Kostikov described from forest soil in Belgium (comp. Fig. 27, Hoffmann *et al.* 2007), but molecular marker are not available. Notably, the fusiform morphology shared by SAG 2477 and *Chlorella* sp. BC4VF9 (http://pediastrum.eeb.uconn.edu/isolates /detail/411) further supports the relatedness of both species.

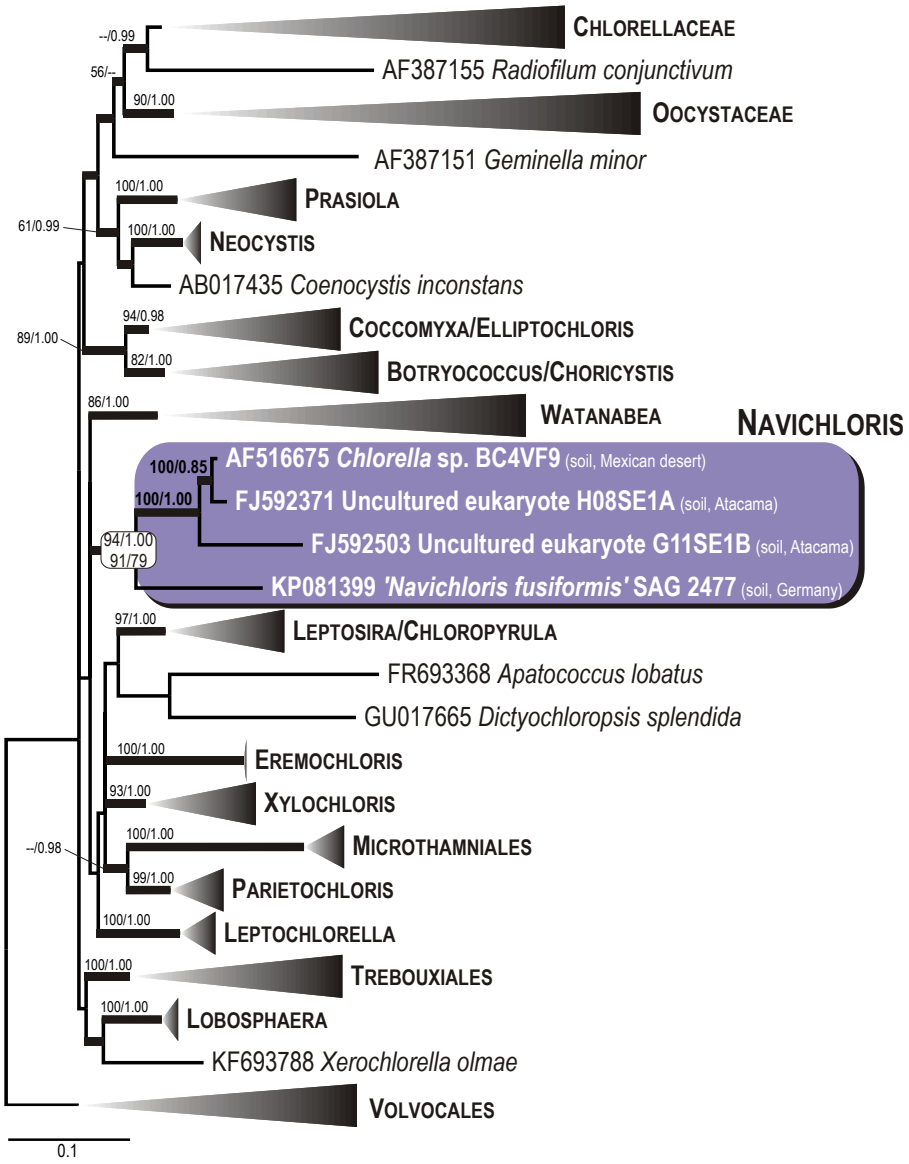

Figure 12. Schematic 18S rDNA phylogeny of the class Trebouxiophyceae. The newly proposed *Navichloris* lineage is highlighted in a violet box. The full 18S rDNA dataset used for the tree computation is identical as in Fučíková *et al.* (2014), Fig. 3. We complemented the dataset by adding three sequences, i.e., KP081399 (SAG 2477; retrieved in this study), FJ592371, FJ592503 (Costello *et al.* 2009); the resulting 18S rDNA dataset comprised 135 sequences. The phylogeny was inferred using RAxML (Stamatakis *et al.* 2008) and statistical support values for the most branches (maximum likelihood/posterior probabilities were computed in the same program and in MrBayes (Ronquist *et al.* 2012), respectively). Additional statistical support values (maximum parsimony and bio-neighbor-joining) for the *Navichloris* lineage were computed in the program SeaView ver. 4 (Gouy *et al.* 2010).

Cultured Green microalgae re-detected in environmental clones

Almost 90% of our cultured species match clones amplified from environmental samples, when considering a ≤ 0.010 SSU-similarity threshold. Even though this similarity level might lack accuracy for species discrimination, it enables to detect species which are closely related; in comparison, Achtman and Wagner (2008) indicates a ≤ 0.013 SSU-threshold for conspecifity in eubacteria. Culture-independent cloning of microalgae from identical sampling sites, conducted by Hallmann *et al.* (in prep.-b), allows to recognize those species, which are re-detectable by both approaches. Due to their frequent occurrence in soils, they might reach high population densities, being key players of soil algal communities (Büdel *et al.* 2009). This applies for several trebouxiophytes (e.g., *Stichococcus* spp., *Coccomyxa* spp., *Chlorella vulgaris*, *Muriella*-like species) and chlorophytes (*Oogamochlamydinia*, *Tatrensinia*, *Jenufa*, *Scenedesmaceae*). Even though comparable cloning studies on soil microalgae are rare, our isolates additionally matched clones from North American soils (*Neocystis*, *Chloroidium, Reinhardtinia*, *Desmotetra;* Lesaulnier *et al.* 2008) and from Dutch historical soil samples (*Muriella*; Moon-van der Staay *et al.* 2006). In multiple cases, clones closely related to our isolates, were amplified from either much dryer or even aquatic habitats, such as *Stichococcus* on marble monuments (Hallmann *et al.* 2013b) and on rocks in the Alps (Horath and Bachofen 2009), *Diplosphaera* in Rio Tinto river (Aguilera *et al.* 2007); *Coccomyxa* (HE617184) in an acidic lake and purification plant in Japan (GenBank acc. no. AB721066), *Lobosphaera* in sulfidic water (Behnke *et al.* 2006) or *Stephanosphaerinia* and *Pseudendocloniopsis* in glacier debris of Alaska (Schmidt and Darcy 2014). Other isolates matched clones amplified from cultured environmental material, such as building stone (Hallmann *et al.* 2013a) and even from aeroplankton in Greece (Genitsaris *et al.* 2011). Remaining cultures were more divergent from all available environmental clones; hence we queried them against selected soil metagenomes deposited in Sequence Read Archive (Wheeler *et al.* 2007). Here we achieved only a ≤ 0.03 match of *Chlorococcum* cf. *minutum* SAG 2479 and *Chlamydomonas* cf. *gerloffii* LH08SW5031 with SSU-V4 pyrotag reads from permafrost metagenome in Sweden (Mondav *et al.* 2014).

Conclusive remarks

Our results suggest that Green microalgae in soils of Central Europe are far from known, being less intensively studied than e.g. desert soil crusts (Lewis and Lewis 2005; Fučíková *et al.* 2014). Even the choice of standard culturing media—supposedly not favoring demanding species (Lukešová 2001; Hoffmann *et al.* 2007)—retrieved a plethora of (phylogenetic) species. The most of them are re-detectable in environmental clone libraries, but some would remain

undiscovered without culturing approach. Particularly high phylogenetic diversity hide in morphospecies known as *Stichococcus* (Nägeli 1849) or *Nannochloris*-like (Henley *et al.* 2004)—consisting of unrelated species which exhibit morphological convergence characteristic for terrestrial Green microalgae (Sharma and Rai 2010). Simplistic morphology of microscopic algae favor their fast dispersal (Sharma *et al.* 2007), however, cosmopolitanism of Green microalgae remains questionable (Lawley *et al.* 2004; De Wever *et al.* 2009). Traditional morphology-based surveys generally proclaim ubiquity of soil Green microalgae (Feher 1948; Starks *et al.* 1981; Ettl and Gärtner 1995). A summary of morphospecies inventory lists collected worldwide (**Fig. S6**) suggests that Green algal communities might share a general morphological composition (i.e., consist of *Stichococcus*-, *Chlorella*-, *Chlamydomonas*-, *Chlorococcum*- and *Klebsormidium*-like morphospecies). Our cultures contribute DNA-based hints indirectly supporting the above mentioned observations—we detected identical ITS2 signatures of *Stichococcus*, *Chlorella* and *Klebsormidium* originating from two different continents (**Fig. 9b**). The ITS2 data further supported the wide distribution of *Muriella* (Kochkina *et al.* 2014) and *Chlorococcum* (GenBank acc. no. FR865591). It remains a major challenge of metagenomics to explore phylogenetic structure and geographic distribution of microscopic algae (Marande *et al.* 2009; Raven 2012; Lie *et al.* 2014). However, not less important is to link OTUs from environmental metagenomes with physical cultures of well characterized species (Richards and Bass 2005; Richards *et al.* 2005; Dickie 2010).

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Chapter 2

Diversity of microscopic green algae (Chlorophyta) in calcifying biofilms of two karstic streams in Germany

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Abstract

For the first time the diversity of microscopic green algae (Chlorophyta) from calcified biofilms of karstic streams was analyzed using a combined approach based on pure cultures, i.e. 18S rRNA gene sequencing and microscopic analyses. Our study focused on two creeks in Germany. A considerable diversity of 34 species of green microalgae comprising three classes, the Trebouxiophyceae, Chlorophyceae and Ulvophyceae, was recovered. The biofilms of both streams were rather different in their species compositions which may reflect that they are exposed to differed hydrochemical conditions. The shallow Westerhöfer creek harbored predominantly Trebouxiophyceae and exhibited higher Mg^{2+} and SO_4^{2-} concentrations. In contrast, the deeper, longer and spatially more heterogeneous Deinschwanger creek harbored numerous species of Chlorophyceae. A lower number of species from the Ulvophyceae were spread on both studied streams. The closest relatives of the identified species were from other freshwater habitats, but mostly from phytoplankton. However, also several species we recovered from freshwater for the first time; so far they have been known from terrestrial habitats only. Less than half of the recovered species could be identified with names at the species level based on high sequence identities with available sequences from named reference strains. Most recovered species could not be identified with names to species level, i.e. they may represent species for which no 18S rRNA gene sequence have become available yet or which have been retrieved for

the first time. The genus *Gongrosira* Kützing, often reported from freshwater tufa-stromatolites, was found to represent most likely a collective morphotype formed by several genera nested within the Ulvophyceae.

Keywords: Chlorophyta; Trebouxiophyceae; Chlorophyceae; Ulvophyceae; 18S rRNA gene phylogeny; karst-water creeks; cultures

Introduction

Many karstic streams in Europe and elsewhere are characterized by calcium carbonate deposits which veneer macrophytes as well as biofilm-covered rock surfaces at the stream bed. These deposits are termed tufa stromatolites, defined as macroscopically laminated benthic microbial deposits produced by precipitation of minerals on organic tissue (Riding 1991). Biomineralization (biological processes) and inorganic precipitation may act together (Ford and Pedley 1996) or photosynthetic CO_2 assimilation by cyanobacteria, eukaryotic algae, and plants may be the primary cause for the carbonate precipitation (Pia 1926; Wallner 1934; Hepperle and Krienitz 1996). Indeed, microsensor studies have demonstrated a photosynthetic control of $CaCO_3$ precipitation for biofilm-covered surfaces, while inorganically driven precipitation prevails e.g. at moss surfaces (Shiraishi *et al.* 2008a; Shiraishi *et al.* 2008b). Microscopic studies revealed the dominance of filamentous cyanobacteria in the calcified biofilms of freshwater karst creeks (Freytet and Plet 1996; Garcia-Pichel *et al.* 2004; Brinkmann *et al.* 2015). But also diatoms, xanthophytes, red algae and other microscopic algae as well as bryophytes and microscopic fungi occur being associated with freshwater stromatolites (Winsborough and Golubić 1987; Heath *et al.* 1995; Freytet and Verrecchia 1998; Bilan and Usov 2001; Brinkmann *et al.* 2015).

In an ongoing larger study the possible roles of photosynthesis and respiration in calcification processes are being studied in detail at two exemplar karstic streams with prominent CO_2-degassing along their course, the Westerhöfer creek and the Deinschwanger creek in Germany. Both tufa-forming streams attain high calcite supersaturation during their course downstream. The Westerhöfer creek, located in Middle Germany in the westerly Harz-foreland (51°45'N, 10°5'E), is 325 m long and less than 2 m wide and has its source in limestones and evaporites of the Middle Triassic Muschelkalk Group. The Deinschwanger creek is located in southern Germany at the western rim of the Franconian Alb (49°23'N, 11°28'E). It is fed by three main springs and a number of side springs, most of them discharging from the Upper Jurassic

Weißjura-Group aquifer. Compared to the Deinschwanger creek the Westerhöfer creek is rich in Mg^{2+} and SO_4^{2-}. Microscopy of biofilm samples from both creeks revealed cyanobacteria and diatoms as the dominant algae (Brinkmann *et al.* 2015), but other micro-algae were found only rarely or not at all. Concurrently with a study on the biodiversity of the cyanobacteria and diatoms from both creeks (Brinkmann *et al.* 2015), cultures of green algae were developed. Interestingly, in the enrichment cultures an unexpected variety of green micro-algae appeared besides numerous cyanobacteria and diatoms. It is not known yet whether there are green algal taxa with strict or even any preference for calcifying biofilms. Their presence in calcifying biofilms may even be entirely given by accident. There is an expectation that the most green algae in calcifying biofilms could originate from soils and other aerial and subaerial habitats. Here we report about the phylogenetic and morphological diversity of these green microalgae.

Materials and Methods

Sampling, culturing and microscopy

Biofilms from both, the Westerhöfer and Deinschwanger creeks, were collected in the spring or early summer (May/June) in 2005–2007. Five sites of the Westerhöfer creek (abbreviated as WB; **Figure A**) and eight sites from Deinschwanger creek (abbreviated as DB) were selected for sampling of apparently algae-dominated biofilms (**Table 1**). For starting enrichment cultures all samples from the WB were pooled together because it is rather short and only a short segment (about 350 m) of the creek was investigated, whereas for the DB the samples from different locations were analysed separately to better reflect its higher habitat heterogeneity. The biofilms were scratched off from stone surfaces using an ethanol-sterilized knife or spatula and transferred to 1.5 ml reaction tubes which were cooled until further processing in the laboratory the following day. A spatula-full of biofilm material was then transferred into different standard liquid growth media. Because our initial main focus was to establish cultures of cyanobacteria and diatoms, growth media

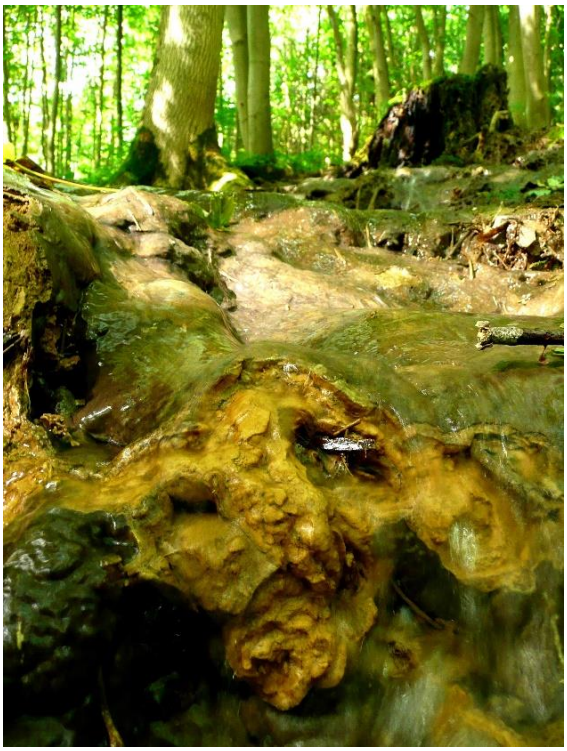


Figure A. Freshwater stromatolites (Westerhöfer Bach)

provoking the development of these algae were used, i.e. BG11, BG11 without citrate, Z, Z 45/4, and ES (http://www.uni-goettingen.de/de/186449.html). Apart from the expected growth of cyanobacteria and diatoms, also intensive green algal growth was observed after about 5–10 days of cultivation. Putative green algal colonies were transferred on 1.5% agar plates with 3N BBM+V medium (http://www.uni-goettingen.de/de/186449.html). After incubation for another 4–8 weeks, all green algal morphotypes were isolated into unialgal cultures by several rounds of streaking on fresh agar plates of 3N BBM+V medium using sterile platinum needles. Finally, the unialgal isolates were microscopically checked for purity and further maintained on agar slants at 18°C under a light/dark regime of 14-h:10-h and a photon flow rate of about 25 µmol photons m^{-2} s^{-1} from white fluorescent bulbs. A total of 77 pure cultures was established, out of which 45 were studied in more detail (**Table S1**). Nineteen of them were made publicly available, i.e. accessioned by the SAG culture collection (Göttingen University, Germany; www.epsag.uni-goettingen.de). For microscopy an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics and an attached ColorView III camera (Soft Imaging System, Münster, Germany) was used. Micrographs were processed using Cell^D image software (Soft Imaging System, Münster, Germany).

DNA extraction, PCR and sequencing

DNA was extracted from all unialgal isolates (**Table S1**) using the Invisorb Spin Plant Kit (Stratec, Berlin, Germany) as recommended by the manufacturer. Nuclear-encoded 18S rRNA genes were amplified using primers NS1 and 18L (Hamby *et al.* 1988). For some strains the 18S and ITS1-5.8S-ITS2 rRNA gene region was amplified using primers NS1 and LR1850 (Friedl 1996). If a culture was suspected to be contaminated by fungi or bacteria, PCR primer 1650R (3'-TCACCAGCACAYYCAAT-5'; pos. 1652-1636 of the 18S rRNA gene sequence of *Chlorella vulgaris* SAG 211-11b, FM205832) which preferentially binds to members of Chlorophyta, was used as the reverse PCR primer. Conditions for PCR and cycle sequencing reactions and the standard set of sequencing primers were as described earlier (Mikhailyuk *et al.* 2008). The newly determined sequences were deposited in GenBank under the accession numbers KF144164-KF144240 (**Table S1**). In addition also the 18S rRNA gene sequences for the following strains were determined as references: *Chlorococcum vacuolatum* Starr SAG 213-8 (KF144189), *Dilabifilum printzii* (Vischer) Bourelly SAG 467-1 (KF144198), *Scotinosphaera gibberosa* (Vodenicarov & Benderliev) Wujek & R.H.Thompson SAG 75.80 (KF144229) and *S. lemnae* (Punčochářová) Wujek & R.H.Thompson SAG 240-1 (KF144230).

Table 1. Distribution of the recovered green algal species at the sampling sites.

Class	Species	DB1 1.19 3	DB2 1.15 3	DB3 1.11 2	DB4 1.09 1	DB5 1.09 1	DB6 1.11 11	DB9 0.45 2	DBS 0.01 4	WB 0.28-0.99 19
Trebouxiophyceae	*Chlorella* sp.*	+	+	+	-	-	+	-	+	-
	Coccomyxa cf. *pringsheimii*	-	-	-	-	-	-	-	+	+
	Coccomyxa cf. *simplex*	-	-	-	-	-	-	-	-	+
	*Elliptochloris subsphaerica**	-	-	-	-	-	-	-	-	+
	Marvania sp.	-	-	-	-	-	-	-	-	+
	*Muriella terrestris***	-	+	-	-	-	-	-	-	+
	Neocystis cf. *mucosa*	-	-	-	-	-	-	-	-	+
	*Stichococcus bacillaris***	-	-	-	-	-	-	-	-	+
	Stichococcus cf. *deasonii*	-	-	-	-	-	-	-	-	+
	*Stichococcus mirabilis***	-	-	-	-	-	-	-	-	+
	Stichococcus sp.1**	-	-	-	-	-	+	-	-	+
	Stichococcus sp.2	-	-	-	+	-	-	-	-	-
	Stichococcus sp.3	-	-	-	-	-	-	-	-	+
	Stichococcus sp.4	-	-	-	-	-	-	-	-	+
Chlorophyceae	*Acutodesmus obliquus***	-	-	-	-	-	+	-	-	-
	Bracteacoccus aerius-relative	-	-	-	-	-	-	-	-	+
	Bracteacoccus sp.	-	-	-	-	-	-	+	-	-
	Chlamydomonas sp.	-	-	-	-	-	-	-	-	+
	Chlamydopodium sp.	-	-	-	-	-	+	-	-	-
	Chlorococcum minutum-relative	-	-	+	-	-	-	-	-	-
	*Chlorococcum sphacosum**	+	-	-	-	-	-	-	-	-
	Chlorococcum ellipsoideum-relative1	-	-	-	-	+	-	-	-	-
	Chlorococcum ellipsoideum-relative2	-	-	-	-	-	+	-	-	+
	Desmodesmus cf. *armatus*	-	-	-	-	-	+	-	-	-
	Monoraphidium terrestre cf. *dybowskii*	-	-	-	-	-	+	-	-	-
	Mychonastes cf. *homosphaera*	-	-	-	-	-	+	-	-	-
	Mychonastes sp.*	-	-	-	-	-	+	-	-	-
	Pseudomuriella cf. *schumacherensis*	-	-	-	-	-	-	-	-	+
	Scenedesmaceae sp.	-	-	-	-	-	+	-	-	-
Ulvophyceae	*Desmochloris* cf. *halophila*	+	-	-	-	-	-	-	-	-
	*Dilabifilum printzii***	-	-	-	-	-	-	-	+	+
	*Hazenia mirabilis**	-	-	-	-	-	-	+	-	-
	*Pseudendocloniopsis botryoides**	-	+	-	-	-	+	-	+	+
	*Pseudendoclonium akinetum**	-	-	-	-	-	-	-	-	+

Legend to Table 1. The recovered species were distributed at eight sampling sites of the Deinschwanger creek (DB) and the pooled sample from the Westerhöfer creek (WB, from five sites). Numbers below sampling sites are the corresponding values [log IAP/KT] of calcite saturation index ($SI_{Calcite}$, for explanation see Arp et al. 2010) and the total number of species recovered per site. Two asterisks next to a species name indicate 100%, a single asterisk 99.9% sequence identity with a reference strain.

Phylogenetic analyses

To search for the closest neighboring relatives of our isolates their sequences were compared to those from reference strains at NCBI (http://www.ncbi.nlm.nih.gov) using BLASTn queries (Altschul *et al.* 1997). Only almost full neighboring 18S rRNA gene sequences were downloaded together with a selection of reference sequences to better represent the green algal classes Trebouxiophyceae, Chlorophyceae and Ulvophyceae as well as additional green algal lineages and aligned using MAFFT, ver. 6 (Katoh and Toh 2008) available online at http://mafft.cbrc.jp/alignment/server/index.html. Three sequence data sets were constructed after

the alignments were manually refined using BioEdit (Hall 1999). The sequence data set of Trebouxiophyceae (**Fig. 1**) contained 162 sequences and was 1766 positions long with 781/562 variable/parsimony informative sites, that of Chlorophyceae (**Fig. 2**, **Fig. 3**) contained 238 sequences and was 1797 positions long with 938/653 variable/parsimony informative sites, and that of Ulvophyceae (**Fig. 4**) 88 sequences and was 1785 positions long with 953/813 variable/parsimony informative sites. The GTR+Γ+I model was selected as the best fitting model of nucleotide substitution for all three sequence data sets as based on the AIC criterion using jModelTest 0.1.1 (Posada 2008). Phylogenetic trees were calculated using maximum likelihood with the program RAxML 7.0.4 (Stamatakis *et al.* 2008) and Bayesian phylogenetic inference with MrBayes 3.2.1 x64 (Ronquist *et al.* 2012). For the latter, two MCMC runs for three million generations each were employed with one cold and three heated chains with trees sampled every 100 generations. Confidence values for the obtained groups (internal edge support) were inferred from the rapid bootstrapping algorithm (100 replicates) as implemented in RAxML (Stamatakis *et al.* 2008) and from Bayesian posterior probabilities using MrBayes 3.2.1 x64 (Ronquist *et al.* 2012). Pairwise sequence similarities from p-distances were determined as an additional measure of the relatedness of our isolates to certain reference strains/sequences under the Kimura 2-parameter model with the program MEGA5 (Tamura *et al.* 2011).

Statistical analysis

The distribution of all major clades of green algae at studied sampling sites was investigated using multivariate ordination by Non-metric Multidimensional Scaling (NMDS); the analysis was conducted in PAST 2.06 (Hammer *et al.* 2001). The input data corresponded to a matrix similar as in **Tables 1 and 2** summarizing the presence/absence of isolates at particular sampling sites as identified by 18S rRNA gene sequence comparisons.

Table 2. Distribution of the recovered phylogenetic clades of Green algae.

		DB1	DB2	DB3	DB4	DB5	DB6	DB9	DBS
		1.19	1.15	1.11	1.09	1.09	1.11	0.45	0.01
		2	2	2	2	2	5	2	4
Trebouxiophyceae	Chlorellaceae clade	+	+	+	-	-	+	-	+
	Choricystis/Botryococcus clade	-	-	-	-	-	-	-	+
	Prasiolales	-	-	-	+	-	+	-	-
Chlorophyceae	Chlamydomonadales	+	-	+	-	+	+	-	-
	Sphaeropleales	-	-	-	-	-	+	+	-
Ulvophyceae	Ulotrichales	-	+	-	-	-	+	+	+
	Ulvales	-	-	-	-	-	-	-	+

Legend to Table 2. Seven phylogenetic clades of Chlorophyta were distributed at the eight sampling sites of the Deinschwanger creek (DB). Numbers below sampling sites are values [log IAP/KT] of calcite saturation index ($SI_{Calcite}$, for explanation see Arp *et al.* 2010) and the total number of clades recovered per site ("phylogenetic diversity").

Results

A total of 77 isolates have been obtained from both creeks (**Table S1**). Phylogenetic analyses of 18S rRNA gene sequences determined for each isolate distinguished 34 distinct lineages supposedly corresponding to species. They were distributed on three classes of green algae, the Trebouxiophyceae (14 species; **Fig. 1**), the Chlorophyceae (15 species; **Fig. 2**, **Fig. 3**) and the Ulvophyceae (5 species; **Fig. 4**). Forty-five isolates have been selected for phylogenetic and microscopic analyses in this paper (**Figs. 1-7, Table S1, Table S2**). The other isolates shared identical (partial) sequences and, therefore, were suspected to represent the same species as the selected 45 isolates. Morphological features of the 34 species (77 isolates) were investigated and are summarized in **Table S2**.

Diversity of Trebouxiophyceae

The recovered Trebouxiophyceae isolates were distributed on four major clades of the class (**Fig. 1**). Those isolates with a *Stichococcus*-like rod shaped morphology (**Fig. 5a**, **Fig. 5b**) were distributed in seven distinct lineages within the *Prasiola* clade. Based on high sequence similarities (99.9 or 100 % as determined from p-distances) and short genetic distances with named reference sequences in the phylogenetic analyses (**Fig. 1**) two closely related isolates, WB13 and WB74 (**Fig. 5a**), were identified as *S. bacillaris* and isolate WB69 as *S. mirabilis* (**Table 1**, **Table S3**). For the other *Stichococcus*-like isolates sequence similarities with named references were lower or no closer named references were available at all. Isolate WB38 was named *S.* cf. *deasonii* as its next named reference was *S. deasonii*. For six isolates which were distributed on four independent lineages of the *Prasiola* clade (**Fig. 1**) no close named relatives were available and, therefore, they were named *Stichococcus* sp. 1 (isolates DB6-27, WB65, and SAG 2407), *S.* sp. 2 (isolate D4-2A), *S.* sp. 3 (isolate SAG 2408) and *S.* sp. 4 (isolate SAG 2406; **Fig. 5b**). Based on morphology all seven lineages may be assigned to *Stichococcus*, but in the 18S rRNA gene phylogenies the genus was paraphyletic with many other coccoid and filamentous members of the *Prasiola* clade (**Fig. 1**). The recovered seven lineages of *Stichococcus*-like Trebouxiophyceae may correspond to seven distinct species, but their assignment to a single genus *Stichococcus* is not adequate. The genus as currently circumscribed may in fact encompass several distinct genera.

The unicellular spherical trebouxiophytes (**Figs. 5c-5f**) represented three distinct lineages within the Chlorellaceae clade (**Fig. 1**). The two isolates RK52 (**Fig. 5c**) and D11-2 (**Fig. 5d**) exhibited relatively large rounded cells with cup-shaped chloroplast and a single pyrenoid with

starch grains attached to it, i.e. the *Chlorella* morphotype (**Table S2**). Both isolates had sequences highly similar to each other and their closest neighbors (99.9 % sequence similarity) were several unidentified *Chlorella* sp. strains. Both isolates represented the most frequently recovered green alga in our study, i.e. there was a total of 12 strains with almost identical 18S rRNA sequences and identical morphology representing the species (**Table S1**, **Table S2**). Resolution within the clade corresponding to the Chlorellales in the 18S rRNA phylogeny was low (**Fig. 1**). Both *Chlorella*-like isolates were distinct from the authentic (type) strain of the genus, *C. vulgaris* SAG 211-11b, by a relatively large genetic distance (**Fig. 1**), but a closer assignment to any genus of Chlorellales was impossible. *Hindakia* was the closest relative (**Fig. 1**), but with a relatively low sequence similarity of 99.7 %.

Two other trebouxiophyte species with spherical cells differed from the *Chlorella* morphotype by their smaller cells with a single band-shaped chloroplast without pyrenoid (**Table S2**; **Fig. 5e**, **Fig. 5f**). They were distributed in two distinct lineages outside of Chlorellales, but within the clade representing the Chlorellales (**Fig. 1**). Two isolates (D6-DB2 and SAG 2390) were assigned to *Muriella terrestris* (**Fig. 5e**) due to low p-distances/high sequence similarities (99.9 and 100 %) with strain ASIB V38 (acc. no. AB012845) which has been isolated from soil (Gärtner 1996). Both our isolates also had the same high sequence similarity with an unidentified strain of *Muriella* AS2-4 from freshwater phytoplankton, acc. no. AY195969 (Fawley *et al.* 2004). Therefore, we assume that both our isolates as well as strains ASIB V38 and AS2-4 represent the same species, *M. terrestris*. The other species, represented by isolate WB67 (**Fig. 5f**), shared high sequence similarities with unidentified *Nannochloris*-like strains which together shared a well-supported monophyletic origin with *Marvania geminata*. Because of the relatively low sequence similarity (97.8 %) with the reference sequence of *M. geminata* our isolate probably represents another yet still undescribed species of *Marvania*.

Other isolated trebouxiophytes exhibited reniform (*Neocystis*-like; **Fig. 5g**), elliptic to nearly spherical (*Elliptochloris*-like; **Fig. 5h**) or elongated to elliptic (*Coccomyxa*-like; **Fig. 5i**) cell shapes. The next relative to isolate SAG 2405, characterized by its mucilaginous colonies and reniform cell shape (**Fig. 5g**), was *Neocystis mucosa* strain KR 1989/14 (acc. no. HM565928), therefore it was termed *Neocystis* cf. *mucosa*. Another *Neocystis* strain, *Neocystis brevis* CAUP D802 (acc. no. HQ287929), was slightly more distant to SAG 2405 and the whole clade of *Neocystis*-like trebouxiophytes was highly supported. Isolate WB5-D1e (**Fig. 5h**) was identified as *Elliptochloris subsphaerica* based on its high sequence similarity of 99.9% with *E. subsphaerica* strain SAG 2202 (acc. no. FJ648518). The same short p-distance/high sequence similarity was with unidentified *Elliptochloris* strain SAG 2117 (acc. no. FJ648515). The whole

clade comprising the three *Elliptochloris* sequences was well supported (**Fig. 1**). Two more species exhibited a typical *Coccomyxa*-like morphology (**Fig. 5i**) and were nested within a well-supported clade consisting of several *Coccomyxa* strains. The one species, represented by the single isolate WB40, was most closely related to strain *Pseudococcomyxa simplex* UTEX 274 (acc. no. FJ648514). The other species was represented by four isolates, retrieved from both creeks (**Table S1**). Out of them for isolate WB28 an almost full 18S rRNA gene sequence was determined and strain *Coccomyxa pringsheimii* SAG 69.80 (acc. no. AY762603) represented its closest neighboring sequence.

Diversity of Chlorophyceae

Isolates assigned to the class Chlorophyceae were distributed in two clades representing the orders Chlamydomonadales and Sphaeropleales (**Fig. 2**, **Fig. 3**). The isolated members of Chlamydomonadales exhibited two morphological types, i.e. *Chlorococcum*-like large spherical cells with a single large chloroplast (**Figs. 6a-6e**), *Chlamydomonas*-like monadoid biflagellated cells (**Fig. 6f**), those of Sphaeropleales four morphological types, i.e. *Scenedesmus*-like elongated cells with acute ends (**Figs. 6h-6i**), *Bracteacoccus*-like spherical cells with numerous discoidal parietal chloroplasts without a pyrenoid (**Figs. 7a, 7b**), *Mychonastes*- or *Nannochloris* like small spherical cells with a single chloroplast without a pyrenoid (**Figs. 7c**, **7d**), and *Monoraphidium*-like fusiform cells.

Chlamydomonadales

The *Chlorococcum*-like isolates were distributed in four distinct clades within the larger and highly supported *Stephanosphaerinia* clade in 18S rRNA gene phylogenies (**Fig. 2**). One clade was formed by isolate RK50 and strain SAG 2402 and their named relative *Chlamydopodium vacuolatum* (acc. no. M63001) and, therefore, both were assigned to the genus *Chlamydopodium*. Another sequence, but from an unidentified strain, *Chlamydomonadaceae* sp. KMMCC:EC-34, was even closer with the former two strains (**Fig. 2**). A second well supported clade included the isolates GRK6-DB5, GRK6-DB6, SAG 2400 and SAG 2401. Except for the latter two, which had almost identical sequences, they were all distant to each other and had sequences from unidentified strains as their next closest relatives. The next named closest relative was *Chlorococcum ellipsoideum* strain UTEX 972 (acc. no. U70586) and, therefore, we named our isolates *Chlorococcoum ellipsoideum*-relatives (**Fig. 3**, **Figs. 6c-e**). *C. ellipsoideum*-relative1, isolate GRK6-DB5, was closer related to *C. ellipsoideum* UTEX 972 than the other three isolates of the clade. A third clade contained a single isolate, SAG 2398, which was within a well-

supported clade together with several named closest relatives (**Fig. 2**). Except for *Chlorococcum sphacosum* SAG 66.80 (which corresponds to the authentic strain of the type of the species), our isolate was morphologically rather distinct from them and, therefore, we assigned isolate SAG 2398 to *C. sphacosum* (**Fig. 6a**; **Table 1**, **Table S2**). Both strains, SAG 2398 and SAG 66.80 (acc. no. JN968580), also shared 99.9% sequence similarity (**Table 1**, **Table S3**). Finally, strain SAG 2399 (**Fig. 6b**) was nested within another distinct and highly supported clade with *C. vacuolatum* SAG 213-8 (acc. no. KF144189) and *C. minutum* ASIB T50 (acc. no. JN968585) as the closest next named relatives (**Fig. 2**). Because it appeared more similar in its morphology to *C. minutum* (**Table S2**) we named strain SAG 2399 *Chlorococcum minutum*-relative. The *Chlamydomonas*-like monadoid isolates RK68 (**Fig. 6f**) and DB6-shared identical (partial) sequences and isolate RK68 was within the well-supported *Reinhardtinia* clade in the phylogenetic analyses (**Fig. 2**). Its closest named next relatives were three species of *Chlamydomonas* and *Volvox carteri*. Because it exhibited solitary cells with a typical *Chlamydomonas* morphology we assigned it to that genus, but could not identify it to species level.

Sphaeropleales

The *Scenedesmus*-like isolates were distributed in three independent lineages (species) of the *Scenedesmaceae* clade (Sphaeropleales; **Fig. 3**). *Desmodesmus* cf. *armatus* was with six isolates among the most frequently recovered green algal species of our study (**Table S1**). Both isolates, RK43 (**Fig. 6g**) and strain SAG 2395, for which almost full 18S rRNA gene sequences were determined, formed a highly supported clade together with named reference strain, *Desmodesmus armatus* CCAP 276/4A (acc. no. FR865727) and, therefore, were assigned the isolates to this species (**Fig. 3**). Isolate D22-6-2B (**Fig. 6h**) shared an identical sequence with reference strain *Acutodesmus obliquus* CCAP 276/49 (acc. no. FR865726) and was assigned to this species. Finally, isolate RK49 (**Fig. 6i**) could not be assigned to a certain genus of the *Scenedesmaceae* clade because it was distinct from all named reference strains of that clade, albeit there was week support for a closer relationship with species of *Acutodesmus* (**Fig. 3**). The three *Bracteacoccus*-like isolates were distributed in two independent clades of the Sphaeropleales representing the genera *Bracteacoccus* and *Pseudomuriella* (**Fig. 3**). The next closest named reference for isolate RK3 (**Fig. 7a**) was *Pseudomuriella schumacherensis* SAG 2137 (acc. no. HQ292768) but with a sequence similarity < 99.9% and, therefore, we named our isolate *P.* cf. *schumacherensis*. Despite their morphological similarities with the *P.* cf. *schumacherensis* isolate the two other *Bracteacoccus*-like isolates, SAG 2403 and DB9-3 (**Fig. 7b**), were in a distinct clade of the 18S

rRNA gene phylogeny, i.e. the *Bracteococcaceae* clade. Strain SAG 2403 had a close named relative, *Bracteococcus aerius* UTEX 1250 (**Fig. 3**), but shared less than 99.9% sequence similarity from p-distances with the latter and, thus, was named *Bracteacoccus* cf. *aerius*. In contrast, isolate DB9-3 had no named closest relative; it was most closely to an unidentified *Bracteacoccus* strain (KF-2011f), but rather distant from *B.* cf. *aerius* (**Fig. 3**). Both *Mychonastes/Nannochloris*-like isolates, RK48 and DB6-29 (**Figs. 7c-d**), were within a single well supported clade, representing the genus *Mychonastes* within the Chlorophyceae (**Fig. 3**). Isolate RK48 had *Mychonastes homosphaera* CAUP H6501 (which represents the authentic strain of the species) as its closest named relative, but with a sequence similarity of less than 99.9% (**Table S3**) and, therefore, was named *Mychonastes* cf. *homosphaera*. *Mychonastes*-like isolate DB6-29 was distant from RK48 and had an unidentified *Mychonastes* strain, Itas 9/21 14-8w (acc. no. AY543066) as closest relative (with 99.9% sequence similarity) and, therefore, was named *Mychonastes* sp. (**Fig. 3**). Several species of *Mychonastes* have recently been described as common members of freshwater phytoplankton (Krienitz *et al.* 2011), but our isolates were more distant to those and, therefore, were not included in our phylogenetic analyses. The *Monoraphidium*-like creek biofilm isolate, strain SAG 2393, was most closely related (with sequence similarity < 99.9%) to two reference strains named *M. dybowskii* within the *Selenastraceae* clade and, therefore, was named *M.* cf. *dybowskii*.

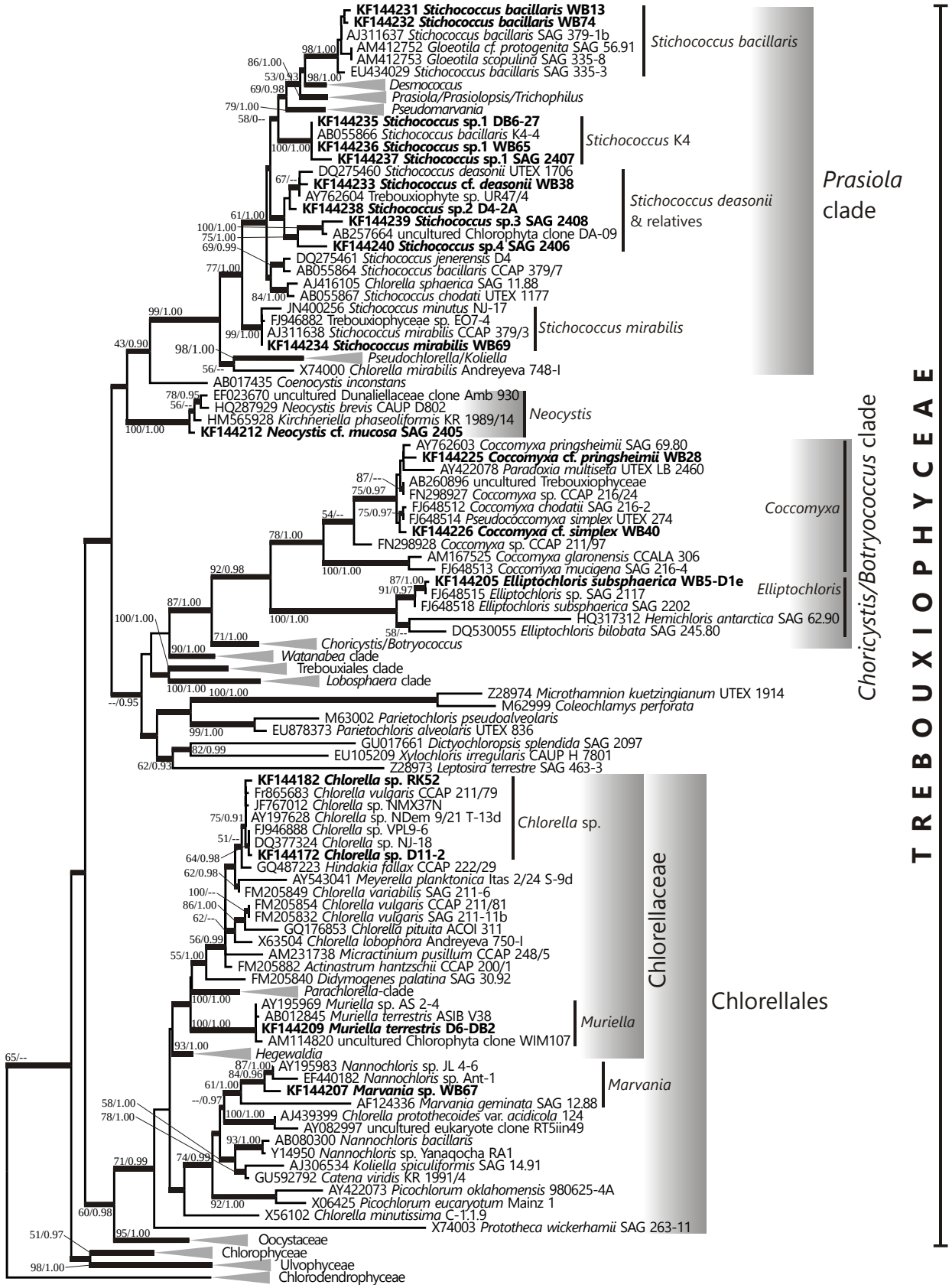


Figure 1. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences of green algal species from the class Trebouxiophyceae isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Trebouxiophyceae as references. Additional members of Oocystaceae (Trebouxiophyceae) as well as other classes of the Chlorophyta, the Chlorophyceae, Ulvophyceae and Chlorodendrophyceae (outgroup), are included for comparisons and to root the tree (for their accession numbers see **Table S4**). Additional outgroups including *Picocystis salinarum* and Nephroselmidophyceae were removed from the figure due to lack of space (all outgroups were the same as in **Fig. 2**, for accession numbers see **Table S4**). Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.

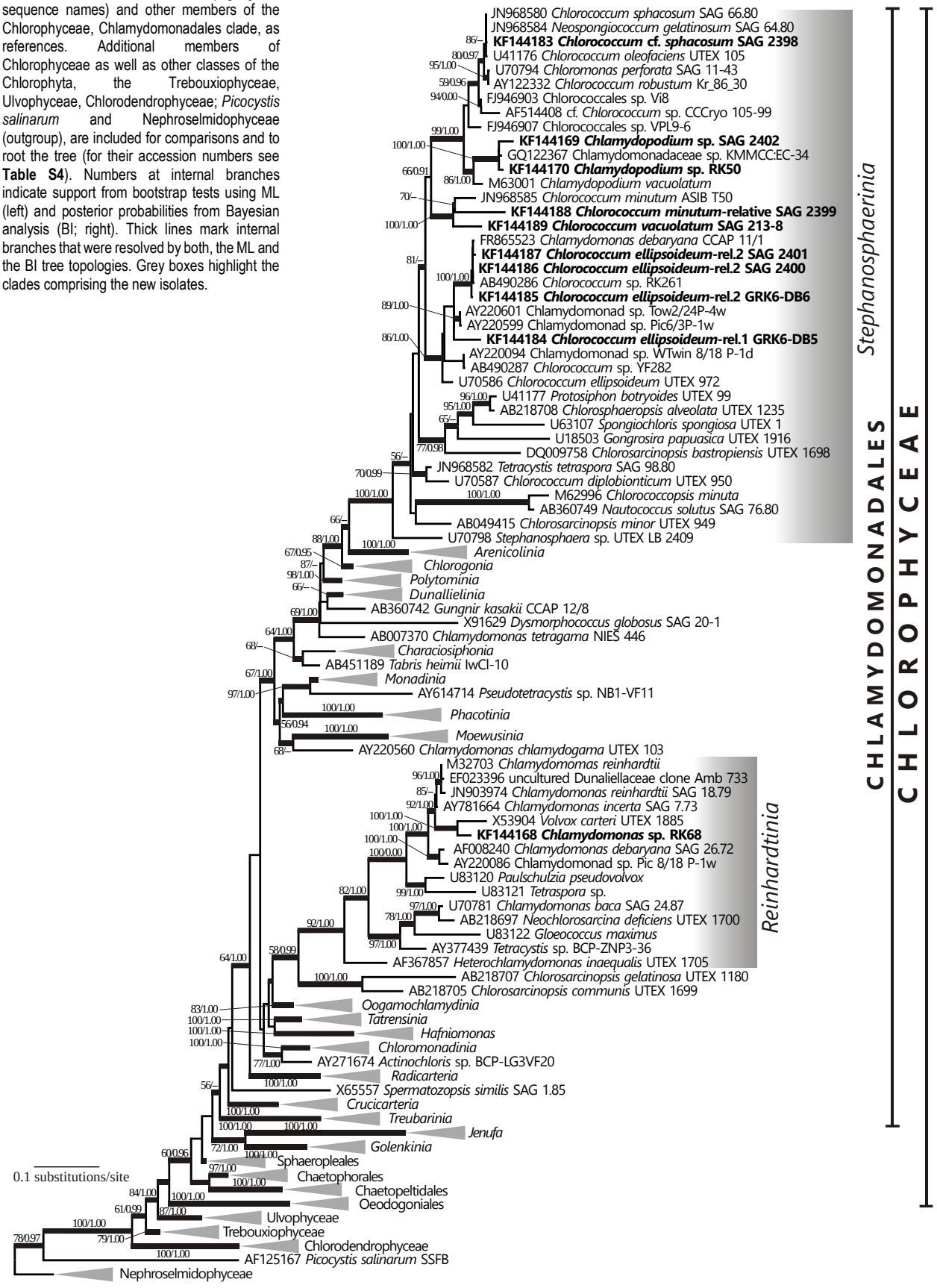
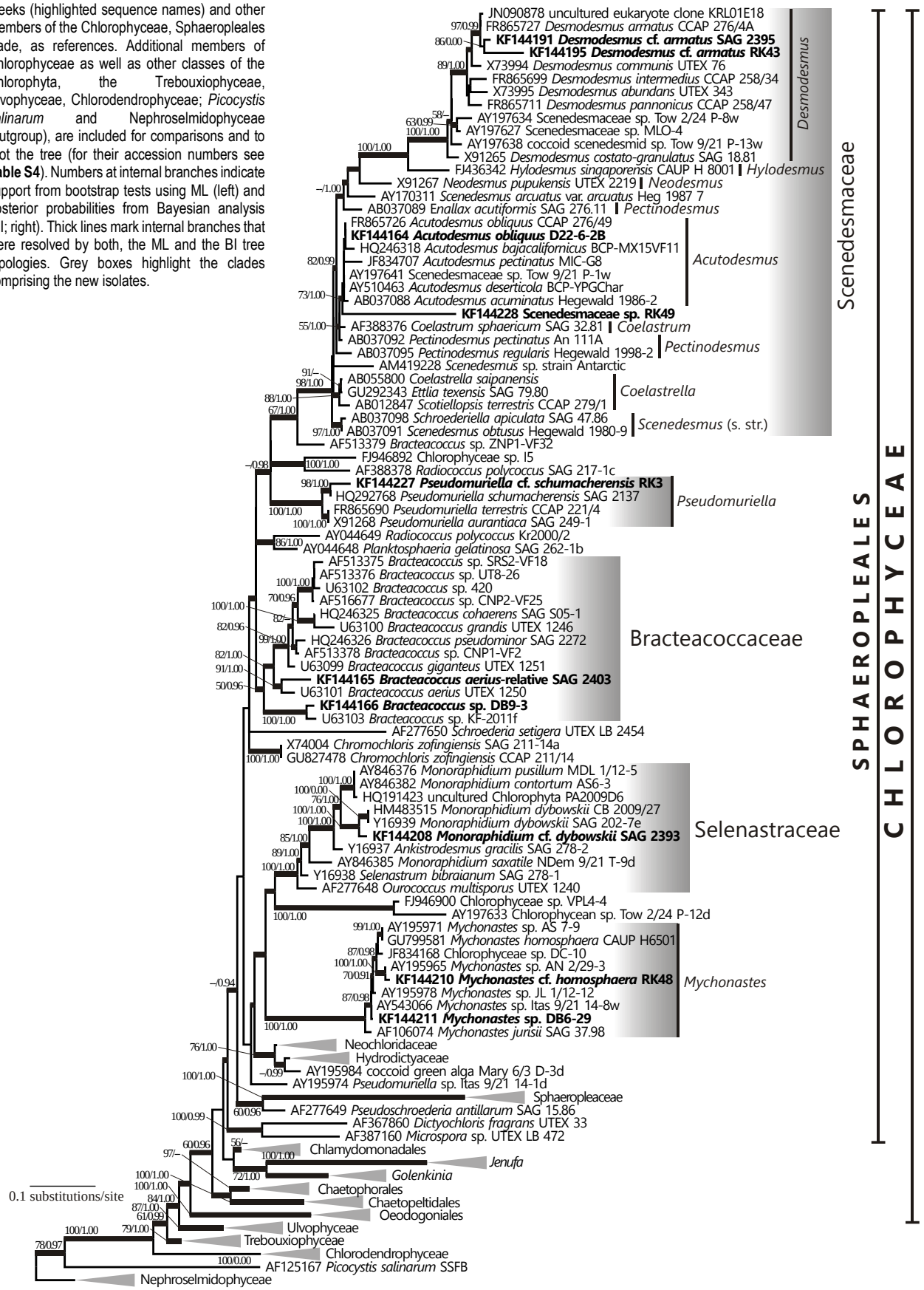


Figure 2. Maximum-likelihood phylogeny of 18S rRNA gene sequences of green algal species from the class Chlorophyceae, Chlamydomonadales clade, isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Chlorophyceae, Chlamydomonadales clade, as references. Additional members of Chlorophyceae as well as other classes of the Chlorophyta, the Trebouxiophyceae, Ulvophyceae, Chlorodendrophyceae; *Picocystis salinarum* and Nephroselmidophyceae (outgroup), are included for comparisons and to root the tree (for their accession numbers see **Table S4**). Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.

Figure 3. Maximum-likelihood phylogeny of 18S rRNA gene sequences of green algal species from the class Chlorophyceae, Sphaeropleales clade, isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Chlorophyceae, Sphaeropleales clade, as references. Additional members of Chlorophyceae as well as other classes of the Chlorophyta, the Trebouxiophyceae, Ulvophyceae, Chlorodendrophyceae; *Picocystis salinarum* and Nephroselmidophyceae (outgroup), are included for comparisons and to root the tree (for their accession numbers see **Table S4**). Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.



Diversity of Ulvophyceae

Five species from creek biofilms were members of the Ulvophyceae. Interestingly, out of them four were of pseudo-filamentous organization, i.e. formed only short filaments and also coccoid stages, whereas only one exhibited a purely coccoid vegetative stage. This was in contrast to our isolates of Trebouxiophyceae and Chlorophyceae which were all of coccoid or monadoid organization. The pseudo-filamentous isolates exhibited characteristic cup-shaped chloroplasts with one or two prominent pyrenoids (**Figs. 7h-i**). Three filamentous species were members of the order Ulotrichales, one belonged together with the coccoid isolate to the Ulvales (**Fig. 4**). Filamentous strain SAG 2396 (**Fig. 7e**) had a rather small genetic distance to *Hazenia mirabilis* UTEX LB 846 (acc. no. AF387156) and, therefore, was assigned to this species (**Fig. 4**.) *Pseudendoclonium basiliense* UTEX 2593 and two species of *Gloeotilopsis* formed up a clade with the former two strains which, however, received only insignificant support values (**Fig. 4**). Another pseudo-filamentous species we recovered was *Pseudendocloniopsis botryoides*; it was with nine isolates (with identical partial sequences and morphology) the second most frequently retrieved green algal species (**Table S1**). As represented by isolate SAG 2394 the close relationship with two available strains of *Pseudendocloniopsis botryoides* was well supported and, therefore, SAG 2394 was assigned to this species (**Fig. 4**). SAG 2394 may also form coccoid stages (**Fig. 7f**), a feature shared with *Planophila laetevirens* SAG 2008 which also was its next closest relative in the 18S rRNA gene phylogeny (**Fig. 4**). Morphology of *P. botryoides* was studied at isolate DB6-19 in more detail (**Fig. 7g**; **Table S2**). The filamentous isolate SAG 2404 (**Fig. 7h**) was a very closely relative to *Pseudendoclonium akinetum* UTEX 1912 and also two species of *Trichosarcina* were their next closest relatives, but these relationships did not receive significant support values (**Fig. 4**). Finally, for another pseudo-filamentous species, *Dilabifilium printzii*, a total of seven isolates was established (**Table S1**); it was the third most frequently recovered green algal species in our study. Unfortunately, we failed to obtain an almost full 18S rRNA gene sequence for these isolates. The partial sequence of isolate WB41 shared 99.9% similarity with the sequence (acc. no. KF144198) of *D. printzii* strain SAG 467-1 and, therefore, we assigned our isolate to this species. Morphological features of isolate WB41 are presented in **Fig. 7i** and **Table S2**. *D. printzii* SAG 467-1 had an independent position within the clade representing the Ulvales in our 18S rRNA gene phylogeny (**Fig. 4**). The single coccoid Ulvophyceae isolate SAG 2397 clearly fell within a subclade of the Ulvales comprising the coccoid genera *Chlorocystis*, *Desmochloris* and *Halochlorococcum* (**Fig. 4**). Strain SAG 2397 had short genetic distances with two strains of *Desmochloris halophila* and, therefore, we

provisionally assigned our isolate to the genus *Desmochloris*. However, a close relationship of strain SAG 2397 with both *D. halophila* strains was not supported in significance tests and *Desmochloris* was paraphyletic in our 18S rRNA gene phylogeny (**Fig. 4**). Consequently, assignment of strain SAG 2397 to a certain species within the subclade of Ulvales remained uncertain.

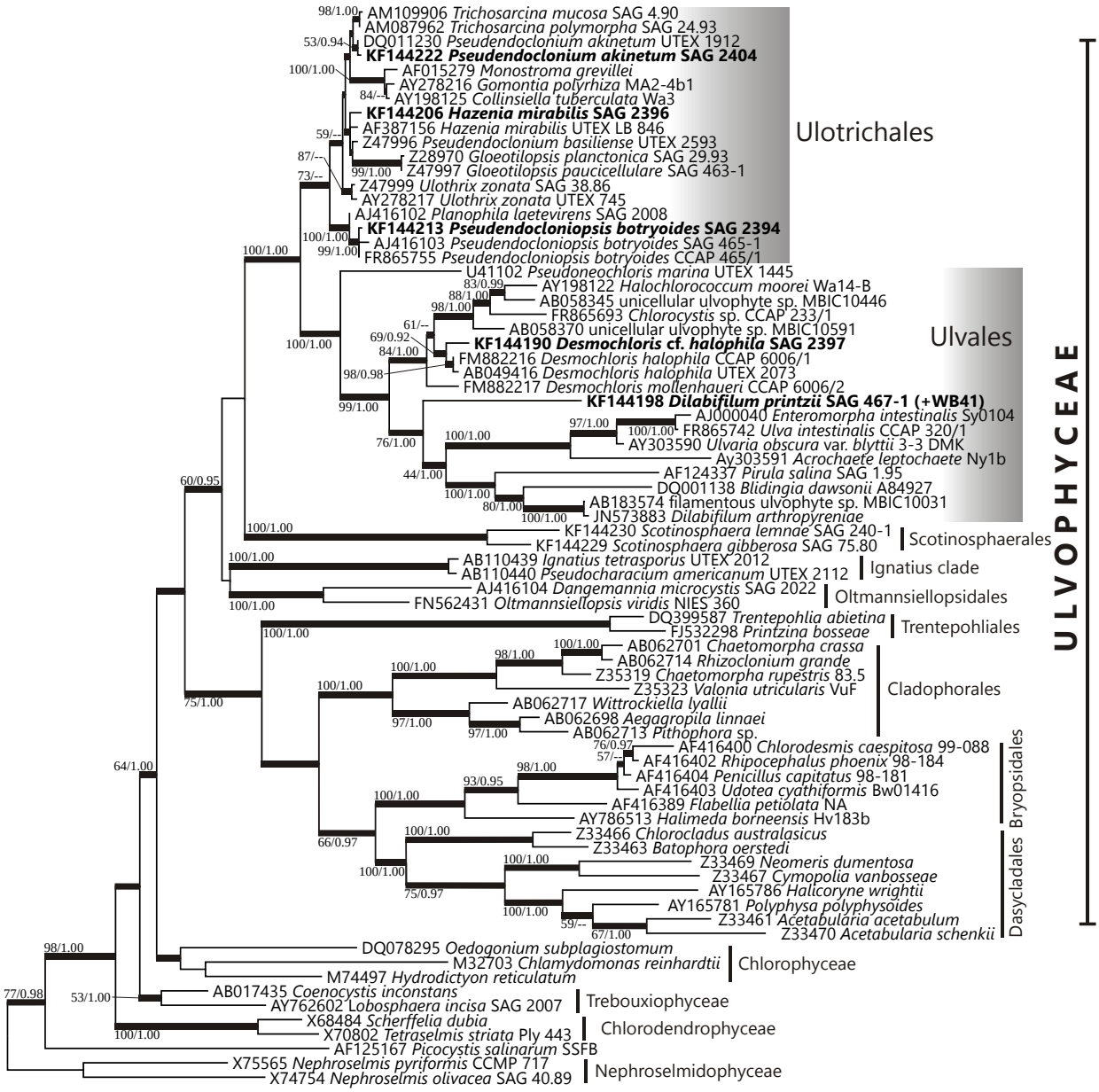


Figure 4. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences of green algal species from the class Ulvophyceae, isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Ulvophyceae as references. Additional members of other classes and lineages of the Chlorophyta are included for comparisons. The phylogeny was rooted with two members of the Nephroselmidophyceae. Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.

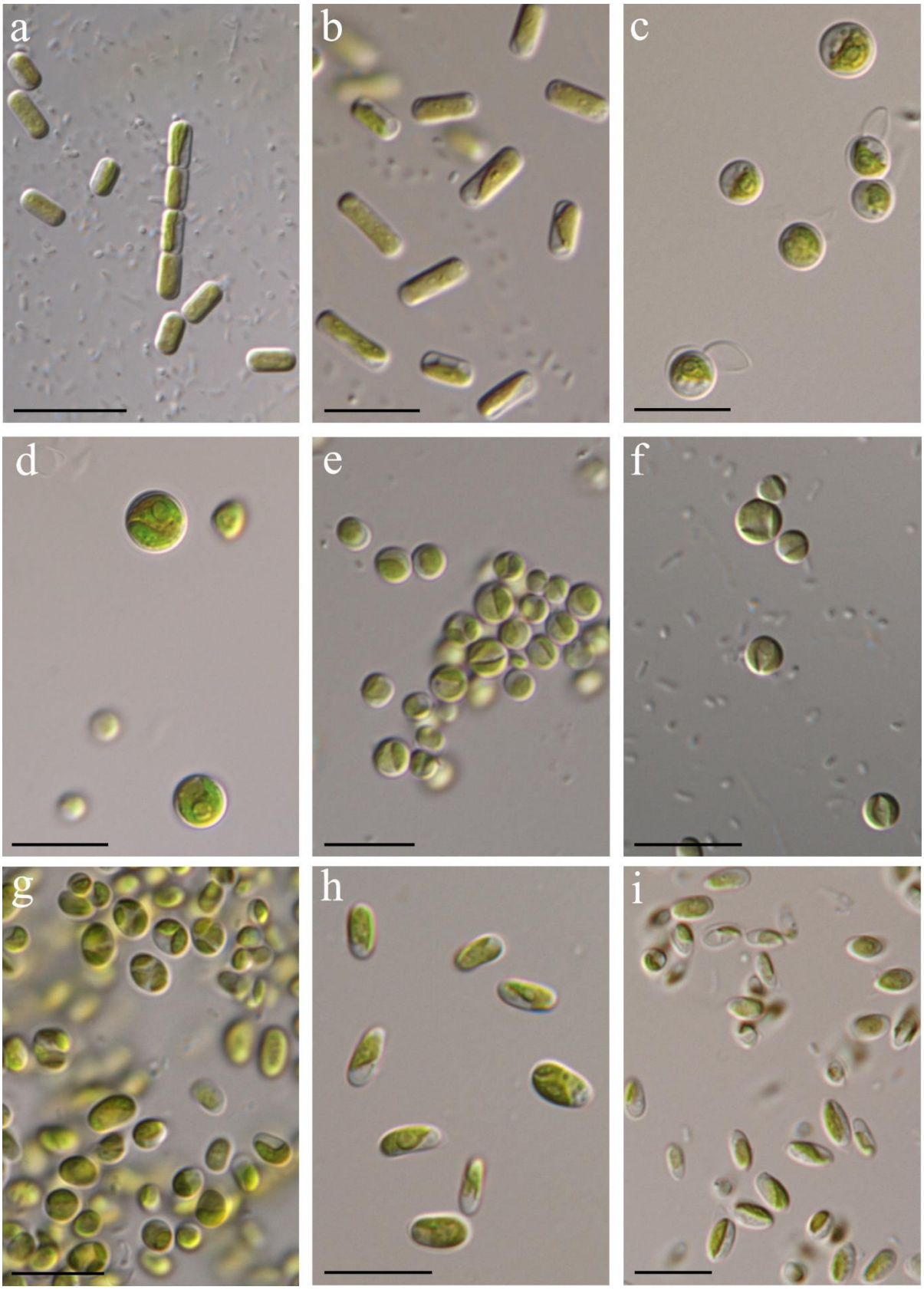


Figure 5. Morphology of green algal isolates of the class Trebouxiophyceae. *Stichococcus bacillaris* isolate WB74 (a), *Stichococcus* sp.4 SAG 2406 (b), *Chlorella* sp. RK52 (c), *Chlorella* sp. D11-2 (d), *Muriella terrestris* D6-DB2 (e), *Marvania* sp. WB67 (f), *Neocystis* cf. *mucosa* SAG 2405 (g), *Elliptochloris subsphaerica* WB5-D1e (h), *Coccomyxa* cf. *pringsheimii* WB32 (i). Scale bars = 10 µm.

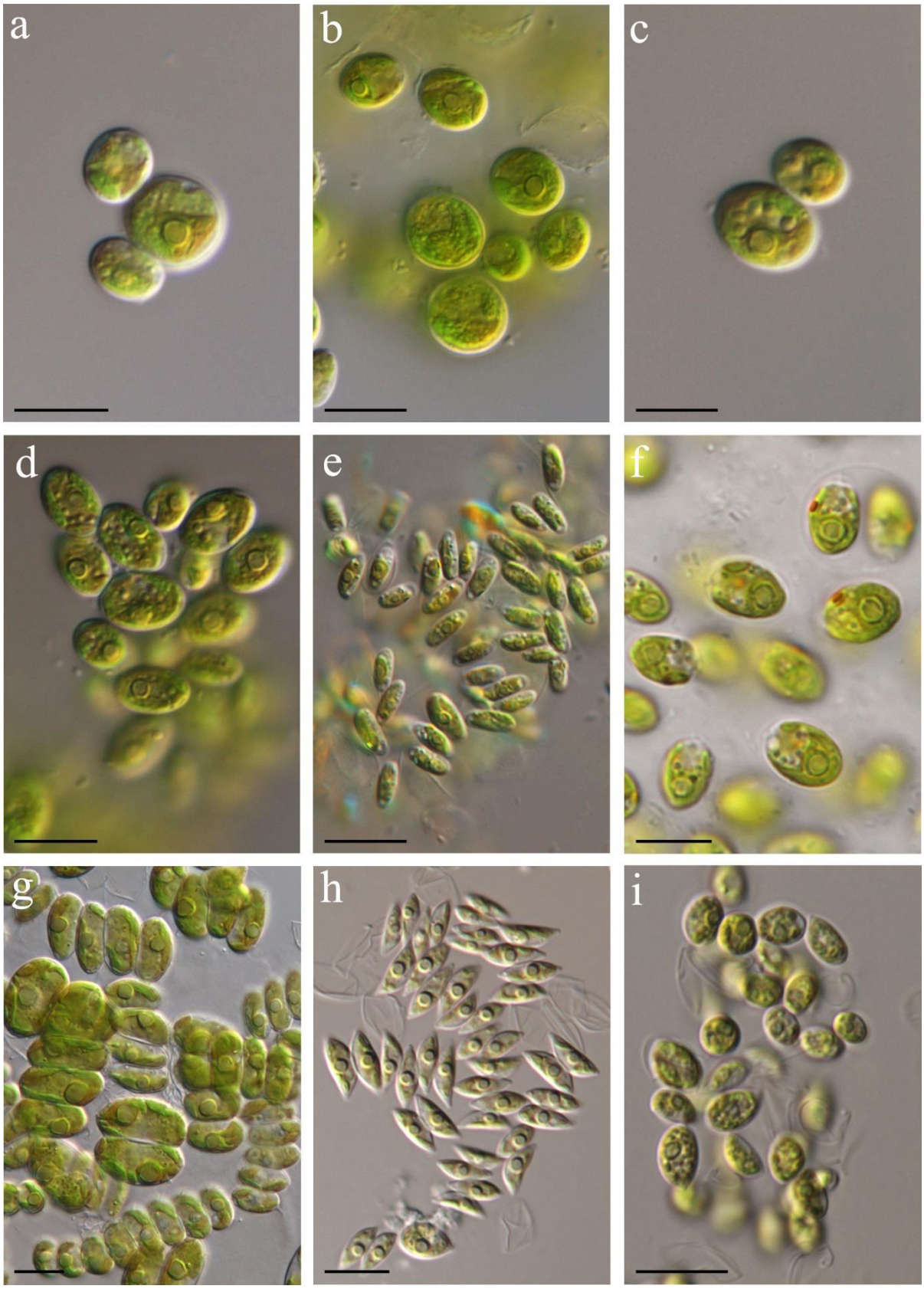


Figure 6. Morphology of green algal isolates of the class Chlorophyceae. Chlamydomonadales (a) – (f); Sphaeropleales (g) – (h). *Chlorococcum sphacosum* SAG 2398 (a), *Chlorococcum minutum*-relative SAG 2399 (b), *Chlorococcum ellipsoideum*-relative1 GRK6-DB5 (c), *Chlorococcum ellipsoideum*-relative2 SAG 2400 (d), *Chlorococcum ellipsoideum*-relative2 GRK6-DB6, zoospores (e), *Chlamydomonas* sp. isolate RK68 (f), *Desmodesmus* cf. *armatus* isolate RK43 (g), *Acutodesmus obliquus* isolate D22-6-2B (h), Scenedesmaceae sp. isolate RK49 (i). Scale bars = 10 µm.

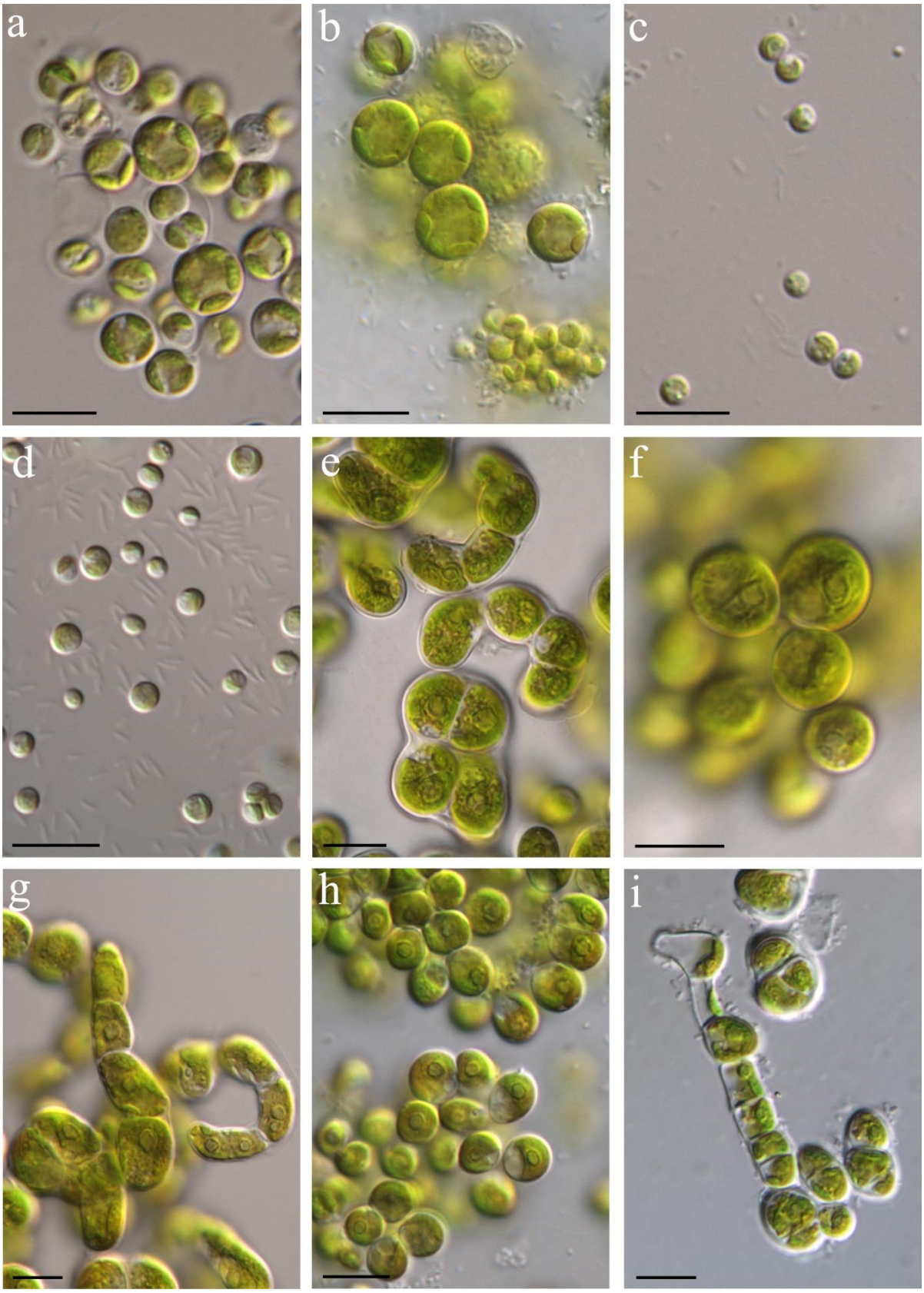


Figure 7. Morphology of green algal isolates of the classes Chlorophyceae and Ulvophyceae isolates. Chlorophyceae, Sphaeropleales (a) – (d); Ulvophyceae (e) – (i). *Pseudomuriella* cf. *schumacherensis* RK3 (a), *Bracteacoccus* sp. DB9-3 (b), *Mychonastes* cf. *homosphaera* RK48 (c), *Mychonastes* sp. isolate DB6-29 (d), *Hazenia mirabilis* strain SAG 2396 (e), *Pseudendocloniopsis botryoides* SAG 2394, coccoid stage (f), *Pseudendocloniopsis botryoides* DB6-19, filamentous stage (g), *Pseudendoclonium akinetum* SAG 2404 (h), *Dilabifilum printzii* isolate WB41 (i). Scale bars = 10 µm.

Discussion

Distribution of the isolates at the sampling sites

In total, we detected nearly the same numbers of green algal species in the Deinschwanger (DB; 21) and Westerhöfer (WB; 18) creeks, regardless of the unequal number of sites sampled per creek (8 at the DB, 5 at the WB; **Table 1**). With respect to calcification the various sampling sites of the DB did not cluster according to high or low calcification when using their species compositions (**Fig. 8**). We conclude that we observed a rather accidental distribution of the biofilm microalgae which is not or only very little influenced by calcification. This is contrast to findings of Brinkmann *et al.* (2015) (this issue) who reported the diversity of cyanobacteria and diatoms of the same creek biofilms as studied here was clearly influenced by $SI_{calcite}$ and pCO_2 which are reciprocally linked. The highest diversity per site (11 species) was detected at site DB6 which was highly calcified (**Table 1**). However, from the other calcified sampling sites (DB1-DB5) only 1-3 species per site could be retrieved in culture. At the spring site DBS with calcification almost absent also just four species were recovered. Two of them, *Coccomyxa* cf. *pringsheimii* and *Dilabifilum printzii* were retrieved only at the DB spring site, but *D. printzii* was recovered from the highly-calcified sites of the WB as well (**Table 1**). In addition, we compared the various DB sites for their "phylogenetic diversity", i.e. whether the species recovered from a certain DB site belong to a single or several phylogenetically distinct clades. With respect to the number of phylogenetic clades retrieved per site, DB6 again was the most diverse site and the spring site DBS was the second most diverse site (**Table 2**). The Chlamydomonadales clade (Chlorophyceae; **Fig. 2**) seemed to have a preference towards the highly calcified DB sites, whereas the Chlorellales (Trebouxiophyceae; **Fig. 1**) and the Ulotrichales (Ulvophyceae; **Fig. 4**) clades were found at the highly calcified sites as well as the spring site of the DB (**Table 2**). With respect to the three different green algal classes detected in both karst-water creeks, the WB was dominated by members of Trebouxiophyceae (12 out of the total of 18 species detected there belonged to Trebouxiophyceae),

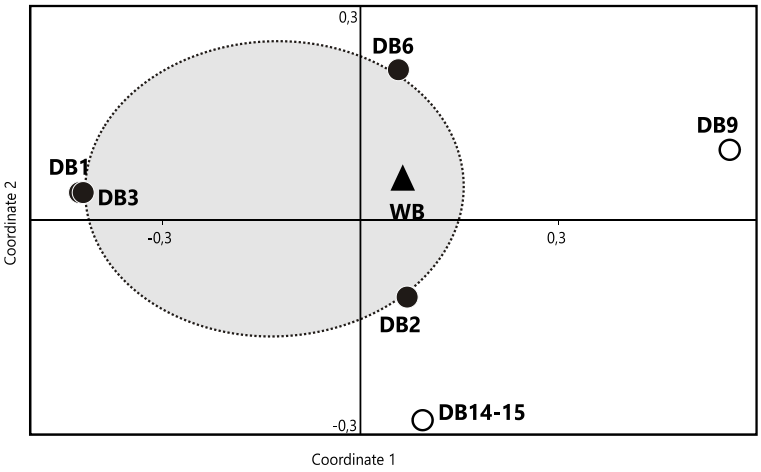


Figure 8. Ordination diagram. Nonmetric Multidimensional Scaling (NMDS) plot showing the grouping of high-calcified sampling sites (black symbols) based on presence/absence of the green algal lineages.

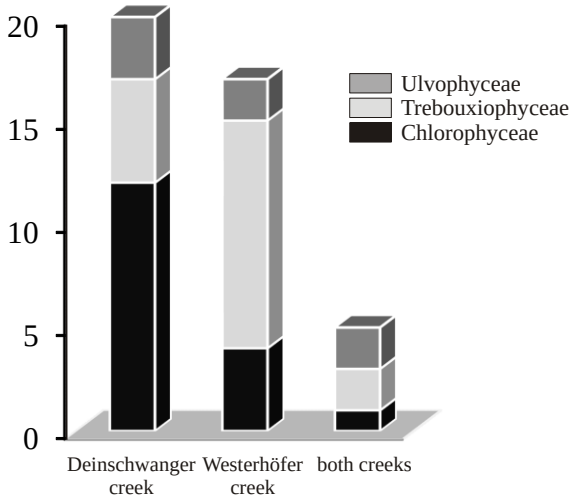


Figure 9. Bar chart showing the relative representation of the three classes of Chlorophyta at the both investigated creeks. Black, Chlorophyceae; light grey, Trebouxiophyceae; dark grey, Ulvophyceae.

whereas the DB predominately harbored members of Chlorophyceae (12 out of the total of 21 species detected there belonged to Chlorophyceae). For the few members of Ulvophyceae no preference to any of the both creeks was found. Interestingly, out of the 34 detected green algal species only six were retrieved from both creeks, i.e. *Coccomyxa* cf. *pringsheimii*, *Muriella terrestris* and *Stichococcus* sp.1 of the Trebouxiophyceae, *Chlorococcum ellipsoideum*-relative2 of the Chlorophyceae, and *Dilabifilum printzii* and *Pseudendocloniopsis botryoides* of the Ulvophyceae (**Fig. 9**). It follows that the species richness of green algae at both creeks was rather different (**Fig. 9**).

Species identification and origins

The molecular phylogenetic diversity of inland freshwater green algae from running waters has been studied for the first time here. In particular, for estimating the diversity of microscopic green algae in epilithic biofilms of running freshwaters, studies are rare and all were exclusively based on morphology so far (for review see Lindstrøm *et al.* (2004) and Veselá (2006)). The majority of existing evidence about the molecular diversity of freshwater green microalgae refers to communities rather different from algal biofilms, e.g. phytoplankton (Fawley *et al.* 2004; Krienitz and Bock 2012). Other molecular data for the diversity of green algae from of running freshwaters have mainly been available from habitats with extreme environmental variables or artificial impact (e.g., Dorigo *et al.* 2002; Aguilera *et al.* 2007; Palacios *et al.* 2008; Baker *et al.* 2009; Aguilera *et al.* 2010). For example, molecular data are available for the periphyton of the extremely cold and remote Antarctica (De Wever *et al.* 2009; Vyverman *et al.* 2010). Compared to freshwater creeks, terrestrial habitats have more extensively been studied by a combined morphological/molecular approach to date (Lewis and Lewis 2005; Flechtner *et al.* 2013).

Sequence comparisons of 18S rRNA genes enable the unambiguous comparisons of new isolates to those previously isolated from other localities and habitats world-wide (De Wever *et al.* 2009; Němcová *et al.* 2011; Hodač *et al.* 2012). Using sequence comparisons most of our

isolates were closely related to those of various other inland freshwaters (**Table S3**). Within the Trebouxiophyceae, these were members of the Chlorellales clade, i.e. *Chlorella* (Bock *et al.* 2010; Bock *et al.* 2011), *Muriella* (Fawley *et al.* 2004) and *Marvania* (Fawley *et al.* 2004; Yamamoto *et al.* 2007). Within the Chlorophyceae, at least the *Scenedesmaceae*, *Selenastraceae* and *Mychonastes*, which we also retrieved in our study, are well known from freshwaters (Krienitz *et al.* 2011; Krienitz and Bock 2012). However, all these genera and families of green algae are known predominantly from major water bodies like lakes or ponds where they inhabit free-floating communities (phytoplankton) and, therefore, were not expected to occur in biofilm assemblages as well. While Trebouxiophyceae and many Chlorophyceae are predominately found in terrestrial and freshwater habitats, Ulvophyceae is a green algal class with a main divergence in marine habitats; it represents the only lineage of Chlorophyta that includes macroscopic sea-weeds (Leliaert *et al.* 2012). In contrast, our isolates were nested within the orders Ulotrichales and Ulvales which are known to occur also in freshwaters and terrestrial habitats as well. Interestingly, the characteristically branched filamentous thalli of the majority of our ulvophycean isolates strongly resembles the morphology of *Gongrosira* which has frequently been reported from tufa formations (Pentecost 1988; Pentecost and Spiro 1990; Freytet and Plet 1991; Johnson and John 1992). Therefore, the name *Gongrosira* may represent a collective morphotype whose members are distributed at least on several distant lineages within the Ulotrichales (Ulvophyceae). Moreover, the only so far sequenced strain of *Gongrosira*, *G. papuasica* UTEX 1916, has been identified as a member of Chlorophyceae (López-Bautista and Chapman 2003).

Using 18S rRNA gene sequence comparisons only for six out of the total of 34 species retrieved in our study had 100% and 99.9% identities with already available sequences from reference strains (**Table 1**, **Table S3**). Therefore, we suspect to have retrieved the same species as previous studies in these cases. However, for none of these species authentic strains were available and, therefore, our identification (species names) may be only as correct as the closest named neighbors (reference strains) of our isolates have been correctly named to species. Except for two reference strains where their origin is not known and *Elliptochloris subsphaerica*, all reference strains for these species have been isolated from various freshwater habitats (**Table S3**) and from a broad range of geographical regions, e.g. Europe, United States and Antarctica. Consequently, our findings support the world-wide distribution of these species. However, it is important to note that most of these freshwater species have been reported from open water bodies as phytoplankton so far, but are recovered by our study from submerged biofilms attached to stones in running waters for the first time. In other cases closest relatives to our isolates were

from terrestrial habitats. Our finding of *E. subsphaerica* is the first record of this species from a freshwater habitat; previously it has been reported only from terrestrial habitats (Ettl and Gärtner 1995). Similarly, our still unidentified isolates of *Stichococcus*, WB38 (*S.* cf. *deasonii*), SAG 2408 (*S.* sp.3) and SAG 2406 (*S.* sp.4), have their closest relatives from terrestrial habitats. Species of *Coccomyxa* have mostly been reported from terrestrial habitats (e.g. tree bark or lichen symbiosis) so far, but were recovered from creek biofilm in our study. The latter three examples indicate that at least parts of the creek biofilm algal communities were probably driven into their habitat through (rain-) flushes from the neighboring soils. For most other biofilm species which so far were reported from phytoplankton only it is still not known whether they can be found in terrestrial habitats as well. *Muriella terrestris* may be an example for a green microalgal species that is known from phytoplankton as well as soil; it was frequently found in the creek biofilms as well.

For 24 species we recovered no identification at the species level was possible, i.e. they were left unnamed despite they could be assigned to a certain genus in most cases. These represent species for which no 18S rRNA gene sequence have become available yet or which have been retrieved for the first time. In this respect we see the importance of molecular-phylogenetic investigations of inland freshwater algal biodiversity, i.e. to extend our knowledge about the distribution of species of green microalgae across various freshwater and terrestrial habitats.

Acknowledgements

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Chapter 3

Phylogenetic analysis of polar *Chlorella* and *Stichococcus* suggests biogeography of airborne microalgae

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Abstract

Chlorella and *Stichococcus* are morphologically simple airborne microalgae, omnipresent in terrestrial and aquatic habitats. The minute cell size and resistance against environmental stress facilitate their long-distance dispersal. However, the actual distribution of *Chlorella*- and *Stichococcus*-like species was so far inferred from ambiguous morphology-based evidence. Here we contribute a phylogenetic analysis of an expanded SSU and ITS2 rDNA dataset including new accessions from terrestrial habitats of polar regions, the temperate zone and the tropics in order to determine whether related species of airborne microalgae show similar patterns in geographic distribution. We found that psychrotolerant strains of *Chlorella* and *Stichococcus* are without exception conspecific (or closely related) with strains originating from the temperate zone. Seven phylogenetic species of polar Chlorellaceae have been uncovered so far with five of them being our new psychrotolerant strains. *Chlorella vulgaris* and *Muriella terrestris* show the widest distribution, including polar and hot desert environments. The morphologically more diversified *Stichococcus*-like lineages were so far detected not only in polar regions and hot deserts, but also in tropical rainforests. However, distinct *Stichococcus* clades exhibit either temperate-polar *or* temperate-tropical distributions. Our data suggest that terrestrial airborne microalgae might exhibit biogeography and are not distributed everywhere.

Keywords: biogeography, *Chlorella*, *Stichococcus*, polar strains

Introduction

Terrestrial species of *Chlorella* (Beijerinck 1893) and *Stichococcus* (Nägeli 1849) are true survivalists among the green microalgae (Chlorophyta). They inhabit biofilms covering natural and artificial subaerial substrates and dwell in soils (Carson and Brown 1976; 1978; Ettl and Gärtner 1995; Sharma *et al.* 2007; Rindi *et al.* 2009). *Chlorella* and *Stichococcus* have been reported by microscopical observations from nearly all soil types, including desert soil crusts in Namibia (Büdel *et al.* 2009), humid tropical soils of India (Ray and Thomas 2012), Central America (Archibald 1972), Oceania (Arvik and Willson 1974; MacEntee *et al.* 1977; Carson and Brown 1978) and polar desert soils in Antarctica (Cavacini 2001; Fermani *et al.* 2007) and the Arctic (Elster *et al.* 1999; Kaštovská *et al.* 2005; Kaštovská *et al.* 2007; Patova and Dorokhova 2008). Langhans *et al.* (2009) recognized species of *Chlorella* and *Stichococcus* as key players for monitoring the succession of biological soil crust formation. Particular attention has been paid to the metabolic facilities of psychrotolerant terrestrial strains of *Chlorella vulgaris* and *Stichococcus bacillaris* (Kvíderová and Lukavský 2005; Shukla *et al.* 2011; Chen *et al.* 2012; Hong *et al.* 2015); both species include strains with the potential for use in biotechnology (Lang *et al.* 2011; Olivieri *et al.* 2011; Cadoret *et al.* 2012; Barreiro *et al.* 2013; Olivieri *et al.* 2013; Goiris *et al.* 2014; Mudimu *et al.* 2014; Safi *et al.* 2014; Sivakumar *et al.* 2014; Slocombe *et al.* 2015). The resistance against environmental stresses connected with metabolic versatility is a common feature of polar microalgae, which have to cope with low temperatures and shortages of nutrients and liquid water (Elster 1999; Elster and Benson 2004; Kvíderová and Lukavský 2005; Sharma *et al.* 2007). An extreme multi-stress resistance was detected in a psychrotolerant cryptoendolithic strain of *Stichococcus* (isolated from Antarctica), which survived an experimental exposure to an Earth orbital space environment (Scalzi *et al.* 2012).

Systematically, *'Chlorella'* is the name for minute coccoid microalgae phylogenetically nested within the Chlorellaceae clade, class Trebouxiophyceae (Huss *et al.* 2002; Krienitz *et al.* 2003; Luo *et al.* 2010; Pröschold *et al.* 2010; Leliaert *et al.* 2012; Krienitz *et al.* 2015). Whereas planktonic Chlorellaceae evolved into distinct forms, e.g., *Micractinium* (Pröschold *et al.* 2010; Krienitz and Bock 2012) or *Actinastrum* (Luo *et al.* 2010; Krienitz and Bock 2012), terrestrial members exhibit morphological convergence, which is characteristic for "true" *Chlorella* (*Chlorella* clade, Bock *et al.* 2011) and for multiple *Nannochloris*-like clades (Henley *et al.* 2004). Morphologically simple *Chlorella*- and *Nannochloris*-like species were repeatedly uncovered in the Antarctic (Gilichinsky *et al.* 2007; Hu *et al.* 2008; De Wever *et al.* 2009).

'Stichococcus' can be as well encountered in the harsh terrestrial and freshwater environments of the Antarctic and Arctic (De Wever *et al.* 2009; Vishnivetskaya 2009; Khan *et al.* 2011).

'Stichococcus' is the common name for multiple rod-shaped species phylogenetically nested within the *Prasiola* clade (Handa *et al.* 2003; Neustupa *et al.* 2007; Novis *et al.* 2008; Eliáš and Neustupa 2009). The *Prasiola* clade represents a trebouxiophyte lineage with the highest morphological diversity within the class (Leliaert *et al.* 2012), which is widely distributed in freshwaters, marine and terrestrial ecosystems (Karsten *et al.* 2005; Rindi *et al.* 2007). Despite their morphological simplicity, *Stichococcus* species veil a considerable phylogenetic diversity, which is still in need of taxonomic revision (Neustupa *et al.* 2007; Sluiman and Guihal 2008; Karbovska and Kostikov 2012a). The rod-shaped *Stichococcus* species are scattered across the whole *Prasiola* clade, but are not intermixed with morphologically distinct lineages such as *Pseudomarvania* (Eliáš and Neustupa 2009) or the thallous Prasiolales (Handa *et al.* 2003; Rindi *et al.* 2004; Rindi *et al.* 2007). SSU-based indications, that the *Prasiola* clade and the Chlorellaceae might represent sister lineages (Handa *et al.* 2003; Fučíková *et al.* 2014), appear to be questionable, while the *Prasiola* clade is undoubtedly nested within the core Trebouxiophyceae (Leliaert *et al.* 2012; Lemieux *et al.* 2014; Turmel *et al.* 2015), plastid genome phylogenies place Chlorellaceae outside the core Trebouxiophyceae (Lemieux *et al.* 2014; Leliaert and Lopez-Bautista 2015; Turmel *et al.* 2015).

Chlorella, *Stichococcus* and a handful of other green algal morphospecies are counted among the so called airborne algae (Sharma *et al.* 2007; Sharma and Rai 2010), which are supposed to be ubiquitous in terrestrial ecosystems (Rindi *et al.* 2009; Rindi *et al.* 2011). However, the existence of limitedly dispersed cryptic species which are morphologically hard to determine, became the rule since the introduction of molecular techniques into phycology (Boenigk *et al.* 2005; Rindi *et al.* 2008; Dal Grande *et al.* 2014; Řídká *et al.* 2014; Ryšánek *et al.* 2014; Škaloud *et al.* 2014a; Škaloud *et al.* 2015). Nevertheless, some species might be true cosmopolitans, as recently affirmed within some lineages of terrestrial green microalgae, e.g., *Coccomyxa* (Darienko *et al.* 2015), *Klebsormidium* (Ryšánek *et al.* 2014) and *Diplosphaera* (Fontaine *et al.* 2012). Remote polar regions like Antarctica and the Arctic provide an opportunity for investigating isolation-by-distance and speciation of microorganisms (Martiny *et al.* 2006; Hahn *et al.* 2015). Pioneering molecular studies on algal diversity in Antarctica found genetic divergence between polar and non-polar species; some detected *Chlorella* and *Stichococcus* species were supposed to be Antarctic endemics (Lawley *et al.* 2004; De Wever *et al.* 2009). Some five years later, the amount of sequenced algal strains and environmental clones has dramatically increased, encouraging us to reconsider whether the Antarctic endemism might be

simply due to a sampling effect. We avoid the difficulty of the algal species concept (Leliaert *et al.* 2012; Leliaert *et al.* 2014); instead, we infer distribution patterns based on monophyletic clades of closely related sequences. By focusing on the polar strains of *Chlorella* and *Stichococcus*, we aim to show that related species of airborne microalgae have similar patterns of geographic distribution.

Materials and Methods

Data sampling and microscopy of the strains

We analyzed new SSU and ITS2 rDNA sequences obtained from the strains, isolates and clones listed in **Table S1** (*Chlorella*-like sequences, accession numbers XY000000-XY000000) and **Table S2** (*Stichococcus*-like sequences, accession numbers XY000000-XY000000). The geographic origin of new sequences from polar/temperate/tropical regions is shown in **Fig. 1**. Isolation of the algal strains and sequencing were described in Hodač *et al.* (2015) while PCR-cloning of the algal sequences from environmental samples was described in Hallmann *et al.* (2013a). Observations of the cultures were conducted using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics and an attached ColorView III camera (Soft Imaging System, Münster, Germany). Cell size measurements (diameter of spherical cells; length/width of elongated cells) were conducted in ImageJ (Schneider *et al.* 2012) and were based on a set of 50-100 cells per culture.

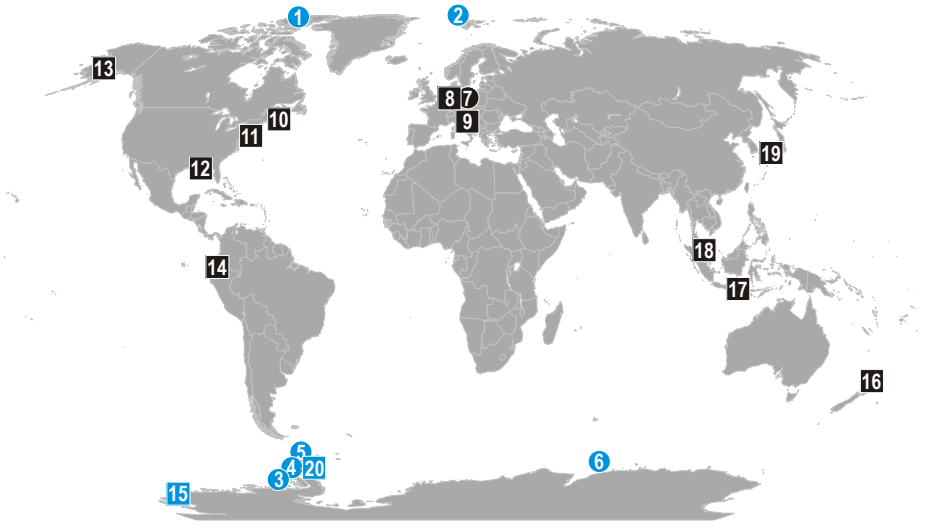

Figure 1. Geographic origin of the new *Chlorella* and *Stichococcus* 18S and/or ITS2 sequences. 1-5: *Chlorella*-like accessions (circles); 1-Canada (Ellesmere Island), 2-Svalbard, 3-Antarctica (Killingbeck Island), 4-Antarctica (Adelaide Island), 5-Antarctica (King George Island), 6-Antarctica (Anchorage Island), 7-German Biodiversity Exploratories. 8-20: *Stichococcus*-like accessions (squares); 8-German Biodiversity Exploratories, 9-Croatia, 10-Canada (New Scotia), 11-USA (Massachusetts), 12-USA (Alabama, Dauphin Island), 13-USA (Alaska), 14-Ecuador (Podocarpus National Park), 15-Antarctica (Victoria Land), 16-New Zealand (Waweira Scenic Res.), 17-Indonesia (Bali, Lake Bratan), 18-Malaysia (Kampong Kuala Jenera), 19-Japan (Taishaku-kyo Gorge), 20-Antarctica (Marie Bird Land). Blue symbols represent polar accessions. Detailed information on sampling sites is listed in **Tables S1, S2**.

18S rDNA phylogenetic analyses

The closest relative sequences to the new accessions were searched for in GenBank using the BLAST algorithm (Altschul *et al.* 1997). New and the GenBank accessions were checked for chimeras using Bellerophon (Huber *et al.* 2004). Two separate 18S rDNA alignments were created, one for *Stichococcus*-like accessions (*Prasiola* clade) and one for *Chlorella*-like (Chlorellaceae) accessions. Both 18S rDNA sequence alignments were computed using MAFFT v.6 (Katoh and Toh 2008) available online. The aligned sequences were checked for possible misaligned positions in BioEdit 7.0.9.0 (Hall 1999). The 18S rDNA alignment, which included the new *Stichococcus* accessions and the closest relatives from GenBank (*Prasiola* clade), contained 100 sequences/1709 positions (276 variable, 149 parsimony informative). Based on the AIC criterion in jModelTest 0.1.1 (Posada 2008), the GTR+Γ+I nucleotide substitution model was selected as the best fitting the *Prasiola* clade. A maximum likelihood phylogeny was computed in RAxML 7.0.4 (Stamatakis *et al.* 2008) under the proposed model and statistical support values were derived from rapid bootstrapping (1000 replicates) in the same program. An alternative maximum likelihood tree search procedure, i.e., quartet puzzling, was applied for the same 18S alignment using Tree-Puzzle 5.2 (Strimmer and Von Haeseler 1996) available at the Mobyle-Pasteur webserver (Néron *et al.* 2009). For this, the GTR+Γ substitution model was applied. To assess the putative age of dichotomies within the *Prasiola* clade, relative node ages were estimated using BEAST v1.8.2 (Drummond and Rambaut 2007; Drummond *et al.* 2012) with 5 000 000 steps (after 500 000 burn-in) under the relaxed molecular clock option. Absolute ages were estimated following De Wever *et al.* (2009) by setting the minimum and maximum age of the Chlorophyta–Streptophyta split at 700 and 1500 Ma. A tree was visualized using FigTree (Rambaut 2007). The 18S rDNA alignment of Chlorellaceae comprised 82 sequences/1668 positions (350 variable, 226 parsimony informative). The J2:G:5 substitution model was proposed by Treefinder (Jobb *et al.* 2004), which was subsequently used for maximum-likelihood tree reconstruction. Confidence values were inferred from 1000 bootstrap replicates in the same program. The resulting tree was visualized using MEGA5 (Tamura *et al.* 2011). For additional statistical support, Bayesian posterior probabilities were computed in MrBayes 3.2.1 x64 (Ronquist *et al.* 2012). We carried out two MCMC runs for three million generations each with one cold and three heated chains under the GTR+Γ+I evolutionary model (parameters were estimated from the data); trees were sampled every 100 generations. To summarize the genetic similarities within the alignments, the sequences were clustered into operational taxonomic units (OTUs) in the program MOTHUR v.1.13.0 (Schloss *et al.* 2009) implementing the 0.00 and 0.01 thresholds under exclusion of the gap positions. For sequence comparisons, p-distances were

computed in MEGA6 (Tamura *et al.* 2013).

ITS2 rDNA phylogeny and secondary structure analysis

First, 63 new ITS2 rDNA sequences were submitted to the BLAST search (Altschul *et al.* 1997) in order to obtain their closest relatives for phylogenetic comparison. Subsequently, an online ITS2 database (Schultz *et al.* 2006; Selig *et al.* 2008; Koetschan *et al.* 2010; Koetschan *et al.* 2012) was used for ITS2 rRNA annotation (Keller *et al.* 2009). Minimum energy secondary structure models were computed from the annotated sequences using RNAstructure 5.3 (Reuter and Mathews 2010). For very closely related sequences, a few were folded in RNAstructure and the rest modelled using homology modelling in the ITS2 database (Wolf *et al.* 2005). Ambiguous models were compared to those computed in the mfold webserver (Zuker 2003). The ITS2 rRNA secondary structures were visualized by Varna 3.8 (Darty *et al.* 2009). Subsequently, two separate alignments of *Chlorella vulgaris* and *Stichococcus*-like sequences and structures were built using 4SALE 1.7. (Seibel *et al.* 2006; Seibel *et al.* 2008). In the same program, compensatory base changes (CBC) were computed between pairs of sequences. The Chlorellaceae ITS2 alignment contained 39 sequences/321 positions (58 variable, 39 parsimony informative). A statistical parsimony analysis was conducted with the very closely related ITS2 sequences of *Chlorella vulgaris* and *C. pituita* using TCS v.1.21 (Clement *et al.* 2000) to generate ribotype networks. The connection limit was set to 95%. The *Prasiola* clade ITS2 alignment contained 56 sequences/584 positions (252 variable, 189 parsimony informative). Based on sequence-structure alignment, a neighbor-joining tree was computed in ProfDist (Müller *et al.* 2004; Friedrich *et al.* 2005; Rahmann *et al.* 2006; Wolf *et al.* 2008) using the GTR+Γ model and bootstrapping (1000 replicates). A splitting structure among closely related ITS2-ribotypes was reconstructed by neighbor-net analysis in SplitsTree4 4.10 (Huson and Bryant 2006), applying uncorrected p-distances and with ambiguities handled as averages. Bootstrap support values for internal splits were calculated with 1000 replicates. The assignation of sequences into ribotypes was computed in DnaSP v.5 (Librado and Rozas 2009).

Results

Polar Chlorellaceae and allies

The analyzed polar *Chlorella*-like strains exhibit two different gross morphologies: 1) *Chlorella*-like spherical to slightly elliptical cells (Ø = 2.8-8.4 µm) bearing one cup-shaped chloroplast with one (**Fig. 2a**) or two (**Fig. 2b**) pyrenoids; 2) *Nannochloris*- and *Muriella*-like spherical cells (Ø

= 2.2-7.3) with a simple parietal chloroplast without a pyrenoid (**Figs. 2d-f**). According to the 18S-based phylogenetic analysis, the polar strains are nested in four different operational taxonomic units (OTUs; corresponding to genera) of the Chlorellaceae (**Fig. 3**). The OTUs 1-4 include further GenBank accessions, which are closely related to our polar strains at the ≥ 99.5% sequence similarity level.

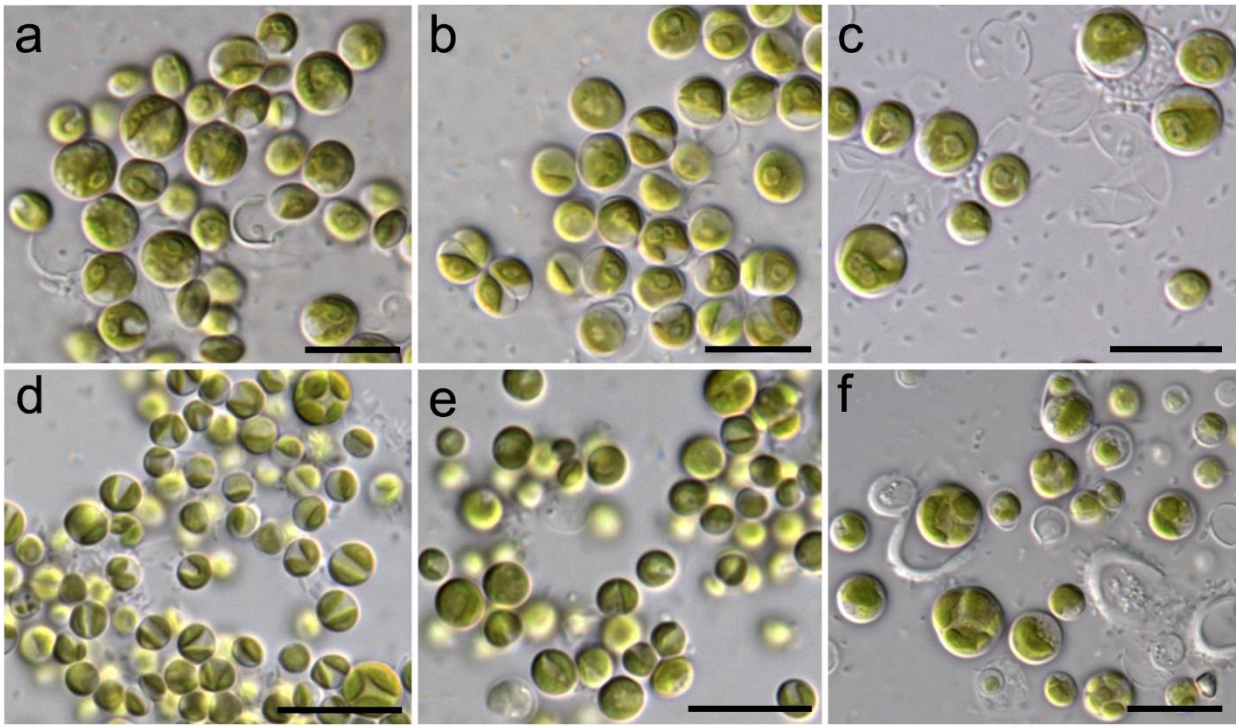

Figure 2. Microphotographs of polar strains of the Chlorellaceae. (a) *Chlorella* cf. *vulgaris* L5 (Ø = 2.8-5.7 µm); (b) *Chlorella* sp.1 N9 (Ø = 2.9-5.4 µm); (c) *Chlorella* sp.2 L3 (Ø = 3.4-8.4 µm); (d) *Muriella* sp. L20 (Ø = 2.2-6.3 µm); (e) *Marvania* relative L15 (Ø = 2.3-5.8 µm); (f) Unidentified Chlorellaceae L24 (Ø = 2.6-7.3 µm). Scale bars = 10 µm.

Chlorella clade

The Antarctic strains L1 and L4 (King George Island) and the Arctic strains L5 (**Fig. 2a**) and L6 (Ellesmere Island) show ≥ 99.5% sequence similarity to the authentic strain *C. vulgaris* SAG 211-11b. The 18S phylogeny suggests divergence between the polar strains and the remaining mostly temperate sequences within OTU3 (**Fig. 3**). We analyzed the observed differences between the polar and temperate strains of *C. vulgaris* using more variable ITS2 (**Fig. 4a**), assigning them into 13 ribotypes (A, B, E-O), exhibiting a total variability of 54 polymorphic nucleotide positions **Fig. S1a**). The ribotypes A-K differ from *C. vulgaris* SAG 211-11b by a few nucleotide polymorphisms (**Fig. S1a**). In contrast, ribotypes L-O are more divergent from *C. vulgaris* SAG 211-11b; they differ from both SAG 211-11b and ribotypes A-K by sharing some identical nucleotide sites with *C. pituita* ACOI 311 (**Fig. S1a**). The ITS2 secondary structure

model (**Fig. S1b**) shows that a considerable amount of nucleotide positions are conserved across all *C. vulgaris* ribotypes (including the polar ribotypes) and *C. pituita* (**Fig. S1b**). Moreover, none of the ribotypes (not even the polar ones) shows any compensatory base changes (CBCs) against *C. vulgaris* SAG 211-11b, but all show at least three CBCs against *C. pituita* ACOI 311 (**Fig. S2a**). Notably, the Antarctic strain L4 (ribotype M) differs in only a single nucleotide from the German isolate LH10HG2067 (ribotype L). Otherwise, the same ribotypes were detected from both aquatic and terrestrial habitats (i.e., ribotypes B, H, I; **Fig. 4b**). Ribotype B is the most common ITS2 variety of *C. vulgaris* in GenBank, including strains isolated from freshwaters in Europe, USA and China.

The Arctic strain N9 (**Fig. 2b**) and the Antarctic strain L3 (**Fig. 3c**) morphologically resemble members of the *Chlorella* clade. Considering the 18S phylogeny, both strains are nested within an unnamed clade (OTU1), which comprises closely related strains from freshwaters of the temperate northern hemisphere and Antarctica. The 18S inference points out the relatedness to the strain *Hindakia fallax* CCAP 222/29. However, the ITS2 phylogeny does not support the close relationship (**Fig. 4a**; **Fig. S2b**), since both strains exhibit considerable genetic divergence from both *Hindakia* species. The ITS2 inference further suggests that N9 and L3 represent two different species, provisionally denominated as *Chlorella* sp.1 and *C.* sp.2, respectively. We detected a remarkably high ITS2 similarity between the *Chlorella* sp.1 strain N9 from the Arctic and the strain KNUA034 from Antarctica, differing by three nucleotide positions (and a one-sided/hemi compensatory base change/hCBC in the helix IV, **Fig. 4c**). The *Chlorella* sp.2 strain L3 has no known close relatives.

***Nannochloris*-like clades**

The *Nannochloris*-like Antarctic strains L20 (**Fig. 2d**) and N5 are nested within OTU3, comprising *Muriella terrestris* ASIB V38 and other terrestrial and aquatic strains of almost identical 18S sequences (**Fig. 3**). The high 18S similarity of the strains L20 and N5 contrasts with their considerable genetic dissimilarity in the ITS2 marker (**Fig. 4**; **Fig. S2c**). The Antarctic *Muriella* sp.1 strain L20 exhibits high ITS2 similarity with the Antarctic clone Ant 8/104 and the isolate LH08SG3009 from a German soil. Strain L20 differs from the isolate LH08SG3009 by one hCBC in the helix III of the ITS2 secondary structure (**Fig. 4d**).

The Antarctic strains L13 and L15 (**Fig. 2e**) clustered within the OTU4 comprising further 18S accessions from Antarctica and the temperate regions (**Fig. 3**). The phylogenetically closest named species are *Marvania geminata* SAG 12.88 and *Nannochloris coccoides* CCAP 251/1b. However, any close relationship to OTU4 (provisionally denominated as *Marvania* relative1) is

supported neither in the 18S nor the ITS2 phylogeny (**Fig. 4a**, **Fig. S2d**).

Strain L24 (King George Island) represents a phylogenetically isolated lineage within the Chlorellaceae, so far without known close relatives. The strain L24 (**Fig. 2f**) exhibits morphology resembling *Nannochloris*-like lineages rather than the true *Chlorella* described above.

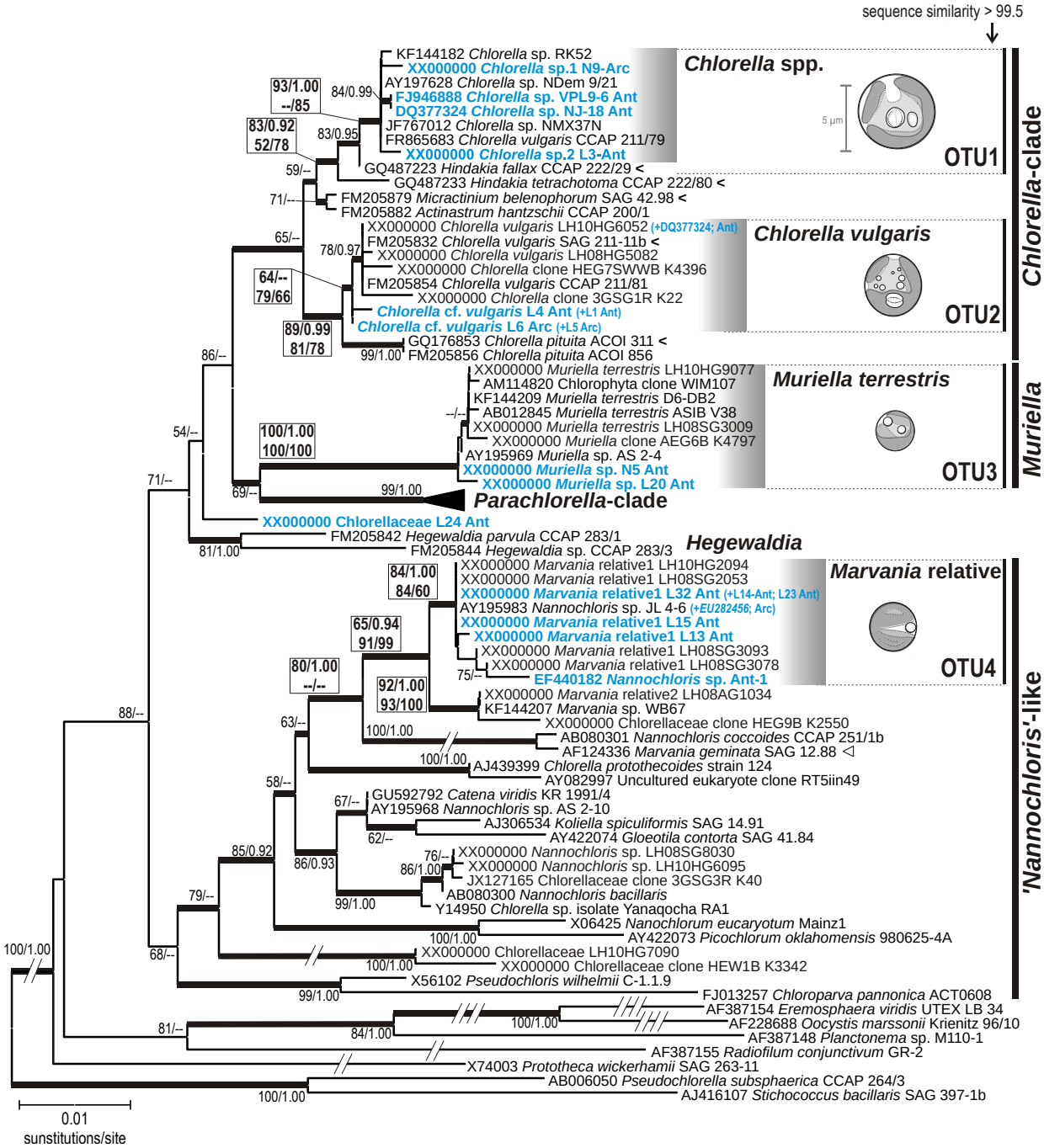

Figure 3. 18S ML-phylogeny of the polar *Chlorella*-like strains and relatives. All polar accessions are highlighted in blue and authentic strains are marked by a '<' sign. The numbers next to branches indicate statistical support values (maximum-likelihood bootstraps (ML)/Bayesian posterior probabilities (BI)); the clades of particular interest were additionally tested via maximum parsimony (MP) and bio-neighbor-joining (NJ) and the statistic support values are given in the following order: ML/BI/MP/NJ. Black bars to the right side give sequence assignations into OTUs at ≥ 99.5% similarity level. Drawings show morphology characteristic for each clade.

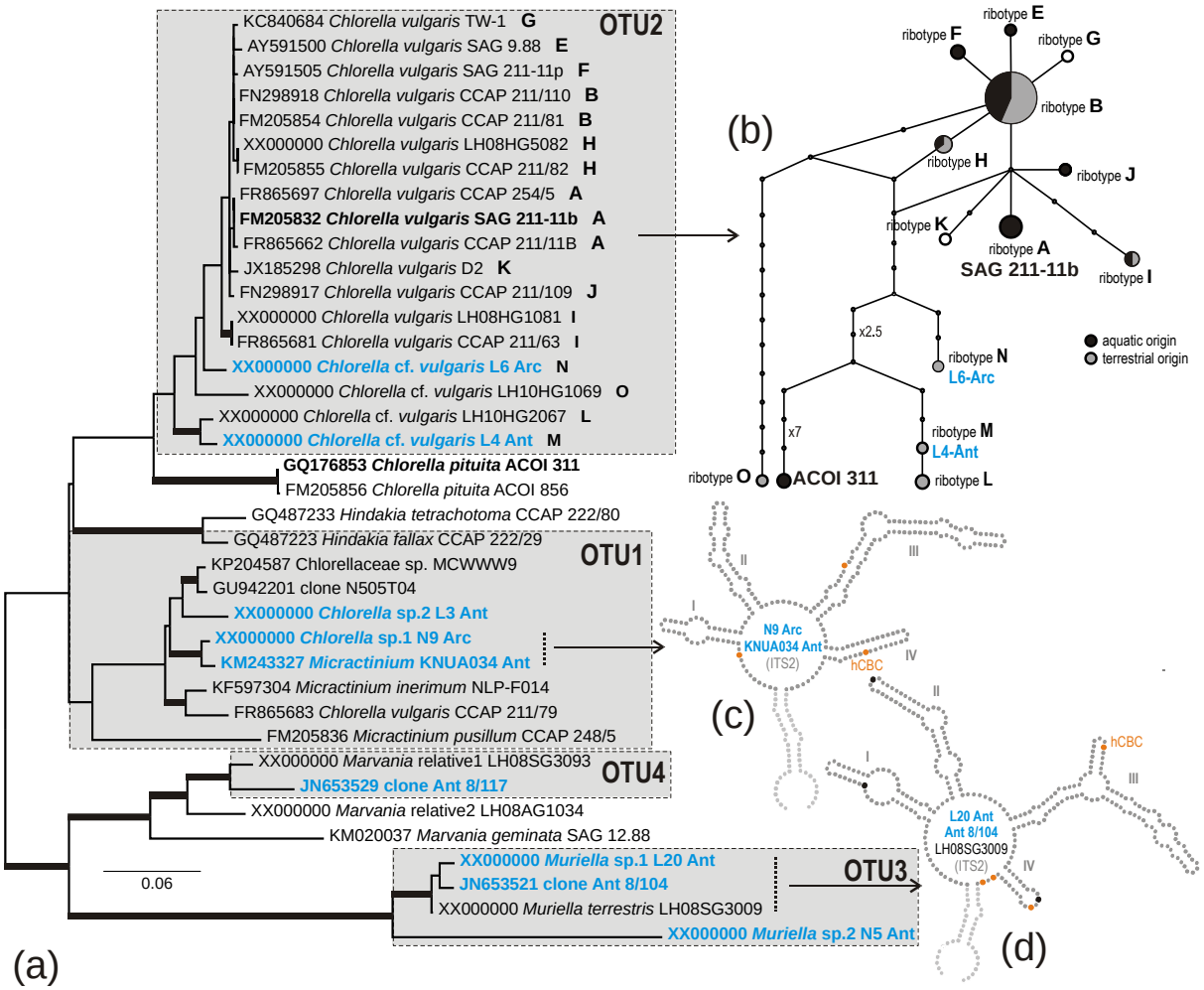

Figure 4. Analysis of ITS2 sequences obtained from polar *Chlorella*-like strains and relatives. (a) An ITS2-based neighbor-joining tree computed from sequences and secondary structures. (b) Ribotype network of *Chlorella vulgaris* accessions assigned to ribotypes A-O. Each circle represents a distinct ITS2 ribotype and its size reflects the number of identical sequences. (c)-(d) Consensual ITS2 secondary structures of closely related accessions: (c) *Chlorella* sp.1, (d) *Muriella* sp. Nucleotide positions within the ITS2 secondary structures are colored as follows: grey dots=conserved positions, orange dots=nucleotide substitutions and black dots=nucleotide deletions.

Stichococcus-like lineages

The newly obtained strains of *Stichococcus*-like species all exhibited characteristic rod-shaped morphology (**Fig. 5a-l**) and varied in both cell length (1.3-10.0 µm) and width (1.0-4.4 µm). Deviations from the rod-shape morphotype—*Diplosphaera*-like cell packages—were observed in the isolate LH08SW1099 (**Fig. 5j**). The *Stichococcus* strains do not substantially differ in chloroplast shape and the detectability of pyrenoids using light microscope is ambiguous. The 18S analysis of all *Stichococcus*-like accessions (**Table S2**) revealed five monophyletic *Stichococcus*-like OTUs of highly similar sequences (≥ 99.5%), i.e., OTU1, OTU3-6 (**Fig. 6**; **Fig S3**) and a further four *Stichococcus*-like OTUs of lower resolution ≥ 99.0%, i.e., *OTU2*, *OTU7-9* (**Fig. 6**; **Fig S3**). The OTUs could be better resolved using more polymorphic ITS2 sequences (**Fig. 7**; **Fig. S4a**) and analyzing their secondary structures (**Fig. 7**; **Fig. S4b**; **Fig. S5**); this approach revealed a total of 12 unambiguously distinguishable monophyletic lineages (**Tab. S3**).

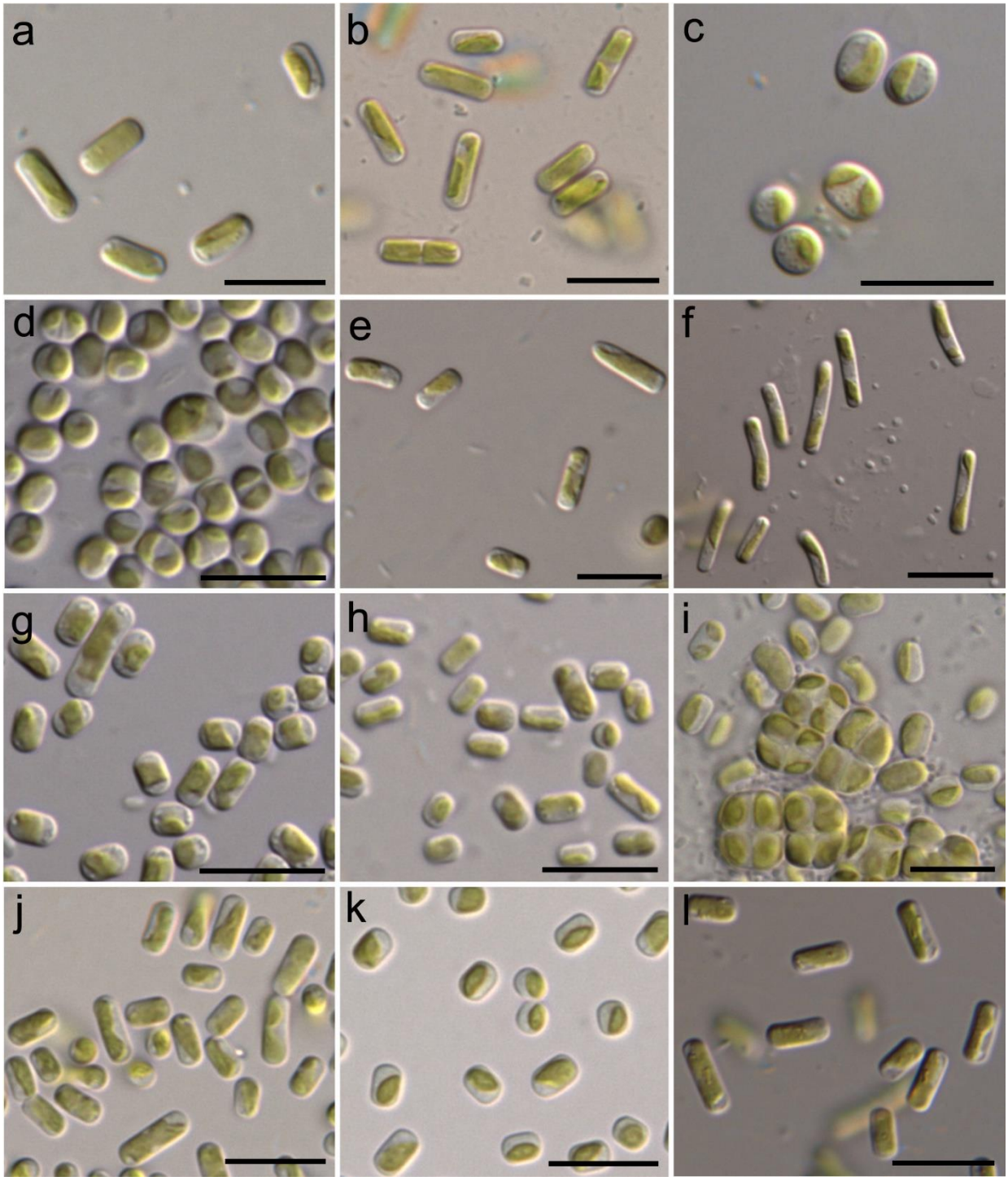

Figure 5. Microphotographs of the *Stichococcus*-like strains isolated from Germany and Ecuador. (a) *Stichococcus* clade2 LH08AW8104 (l = 4.0-10.0 µm; w = 1.8-3.2 µm); (b) *Stichococcus* clade7 LH10HG2063 (l = 4.5-8.7 µm; w = 1.8-2.8 µm); (c) *Diplosphaera* sp. LH08HW8075 (l = 3.2-6.2 µm, w = 1.9-4.4 µm); (d) *Diplosphaera* sp. KS120SM6L (l = 1.3-4.9 µm, w = 1.9-3.4 µm); (e) *Pseudostichococcus* sp. LH08SW8044 (l = 3.4-9.8 µm; w = 1.6-3.3 µm); (f) *Stichococcus* clade1 SAG 2481 (l = 3.1-8.7 µm; w = 1.6-2.9 µm); (g) *Stichococcus* clade1 KS075SM6T (l = 2.9-6.3 µm; w = 1.5-3.0 µm); (h) *Stichococcus* clade3 KS106CL6T (l = 2.5-5.0 µm; w = 1.5-2.4 µm); (i) *Stichococcus* clade5 LH08SW1099 (l = 3.0-6.3 µm; w = 1.0-4.2 µm); (j) *Stichococcus* clade5 KS305SM6L (l = 3.3–6.8 µm; w = 1.4–3.2 µm); (k) *Stichococcus jenerensis* KS126SM6L (l = 1.9-5.0 µm; w = 1.6-3.2 µm); (l) *Stichococcus* clade4 SAG 2406 (l = 4.5-8.2 µm; w = 1.7-2.7 µm). Scale bars = 10 µm.

***Stichococcus*-like clades including polar species**

The polar *Stichococcus*-like accessions (obtained from GenBank; **Table S2**) represent at least three different phylogenetic lineages, here provisionally designed as *"OTU2 Stichococcus deasonii* & allies*"*, *"OTU8 Diplosphaera"* and *"OTU9 Pseudostichococcus"* (**Fig. 8**; **Fig. S3**).

OTU2 consists of the authentic strain *Stichococcus deasonii* SAG 2139 (Alabama, USA) and further accessions originating from terrestrial habitats (**Table S2**). *OTU2* is poorly resolved in the 18S phylogenies (**Fig. 6**; **Fig. S3**). The ITS2 analyses suggest that, apart from *Stichococcus deasonii*, *OTU2* consists of another three different species, here provisionally named as *Stichococcus* clade2 (**Fig. 5a**), *S.* clade6, and *S.* clade7 (**Fig. 5b**). *S.* clade2 includes accessions from Antarctica (e.g., HM490287), differing from its European relatives (e.g., SAG 2482) by 6-9 nucleotide positions in ITS2 (**Fig. S5**). Another two Antarctic strains cluster within *OTU2*: *S. bacillaris* NJ-10 and *S. bacillaris* s3, but the 18S data do not provide sufficient resolution to assign them into any known species. *Stichococcus deasonii* is the only lineage within *OTU2* which comprises accessions from the tropics (e.g., clone KSK870SM6T; **Fig. S5**).

OTU8 Diplosphaera is a phylogenetically heterogeneous clade consisting of multiple lineages of *Stichococcus*-like (*S. chodati* UTEX 1177), *Diplosphaera*-like (*D. mucosa* SAG 48.86) and *Chlorella*-like (*C. sphaerica* SAG 11.88) species, recognizable in the 18S and/or ITS2 phylogenies, yet without sufficient resolution or statistical support. The Antarctic strain *Diplosphaera mucosa* SAG 48.86 is the only known polar member within *OTU8*. Further accessions within the clade originate from Central Europe (isolate LH08HW8075; **Fig. 5c**; **Fig. 6**) the tropics (isolate KS120SM6L; **Fig. 5d**; **Fig. 6**) and North-American deserts (e.g., *Stichococcus chlorelloides* BCP-CNP2-VF11B). *OTU8* is the only known *Stichococcus*-like clade that includes species from hot deserts (**Fig. 8**).

OTU9 Pseudostichococcus represents a phylogenetically isolated clade of *Stichococcus*-like (*S. mirabilis* CCAP 379/3, *Pseudostichococcus monallantoides* SAG 380-1) and *Desmococcus*-like (*D. spinocystis* SAG 2067) species (**Fig. 7**; **Fig. 8**; **Fig. S3**; **Fig. S5**). The clade consists of two strains isolated from Antarctica: Trebouxiophyceae sp. EO7-4 and *Stichococcus minutus* NJ-17. All other accessions within *OTU9* originate from terrestrial (e.g., *Pseudostichococcus* sp. LH08SW8044; **Fig. 5e**) and aquatic habitats of the temperate zone while no tropical relatives are known so far.

***Stichococcus*-like clades including tropical species not related to polar allies**

We identified four clades consisting of tropical and temperate species and designed them provisionally as: "OTU1 *Stichococcus* clade1", "OTU3 *Stichococcus* clade3", "OTU5

Stichococcus clade5" and *"OTU7 Stichococcus jenerensis"* (**Table S2**).

OTUs 1, 3 and 5 are statistically highly supported in both the 18S (**Fig. 6**) and ITS2 (**Fig. 7**; **Fig. S5**) phylogenies and represent three undescribed *Stichococcus*-like species. Few nucleotide differences in the ITS2 within the clades and no compensatory base changes further support close relationships among the tropical and European accessions. OTU1 *Stichococcus* clade1 consists of strains isolated from Germany (SAG 2481; **Fig. 5f**) and Ecuador (KS075SM6T; **Fig. 5g**). Another German strain KS075SM6T differs from KS075SM6T by only 10 nucleotide positions within ITS2 and exhibits no compensatory base changes (**Fig. S5**). OTU3 *Stichococcus* clade3 includes the European strain SAG 2408 which differs by only three ITS2 nucleotide positions from the tropical isolate KS106CL6T (**Fig. 5h**). OTU3 further consists of 18S accessions sampled in the Swiss Alps and Yellowstone NP (**Fig. 6**; **Table S2**). OTU5 *Stichococcus* clade5 is a terrestrial lineage consisting of the European isolate LH08SW1099 (exhibiting both *Stichococcus*- and *Diplosphaera*-like morphology; **Fig. 5i**) and the Ecuadorian isolate KS305SM6L (**Fig. 5j**). The tropical isolate differs by four ITS2 nucleotide substitutions from the isolate KP09AW1004 originating from Germany. OTU5 might be distributed as well in Hawaii, since the accession KM462543 shows a high 18S sequence similarity (99.46%) to the members of *Stichococcus* clade5.

OTU7 Stichococcus jenerensis contains the authentic strain *S. jenerensis* SAG 2138, which is the only *Stichococcus*-like species described from the tropics. The ITS2 data suggest a pantropical distribution of the clade: the Ecuadorean isolate KS126SM6L (**Fig. 5k**) differs by 13 ITS2 nucleotides from the Southeast-Asian strain SAG 2138, and exhibiting no CBCs (**Fig. S5**). *OTU7* further consists of temperate terrestrial and even marine accessions (**Fig. 6**; **Table S2**).

Stichococcus-like clades from the temperate zone

Two highly supported clades of terrestrial and aquatic *Stichococcus*-like species are known so far only from the temperate zone: "OTU4 *Stichococcus* clade4" and "OTU6 *Stichococcus bacillaris*". OTU4 contains the *Stichococcus*-like strain SAG 2406 (**Fig. 5l**) and further accessions (sampled in Germany; **Table S2**) with almost identical 18S sequences (**Fig. 6**). However, the ITS2 data point out a cryptic species diversity, since we detected one CBC between strain SAG 2406 and the isolate LH08SG1073 (**Fig. S5**). The strain CCALA 906 from Svalbard (KF355941) is the closest polar relative of OTU4, yet with low 18S similarity (98.7%). OTU6 includes multiple *Stichococcus* strains (e.g., *S. bacillaris* SAG 379-1b; *S. chloranthus* SAG 379-2) and strains morphologically assigned to *Gloeotila* (*Gloeotila scopulina* SAG 335-8; *Gloeotila* cf. *protogenita* SAG 56.91). OTU6 accessions originate from freshwaters (**Table S2**), soil (clone

HEW1B K3375; **Fig. 6**) and even the Southeast Pacific (KF899844). The ITS2 data suggest a close relationship between the OTU6 *Stichococcus bacillaris* and the *Desmococcus* clade (**Fig. 7**; **Fig. S5**), which consists of cell packages-forming/filamentous terrestrial species (distributed in the polar and temperate regions; **Fig. 8**). OTU6 and *Desmococcus* are sister lineages in both the 18S and ITS2 phylogenies and differ by two CBCs in the ITS2 secondary structures.

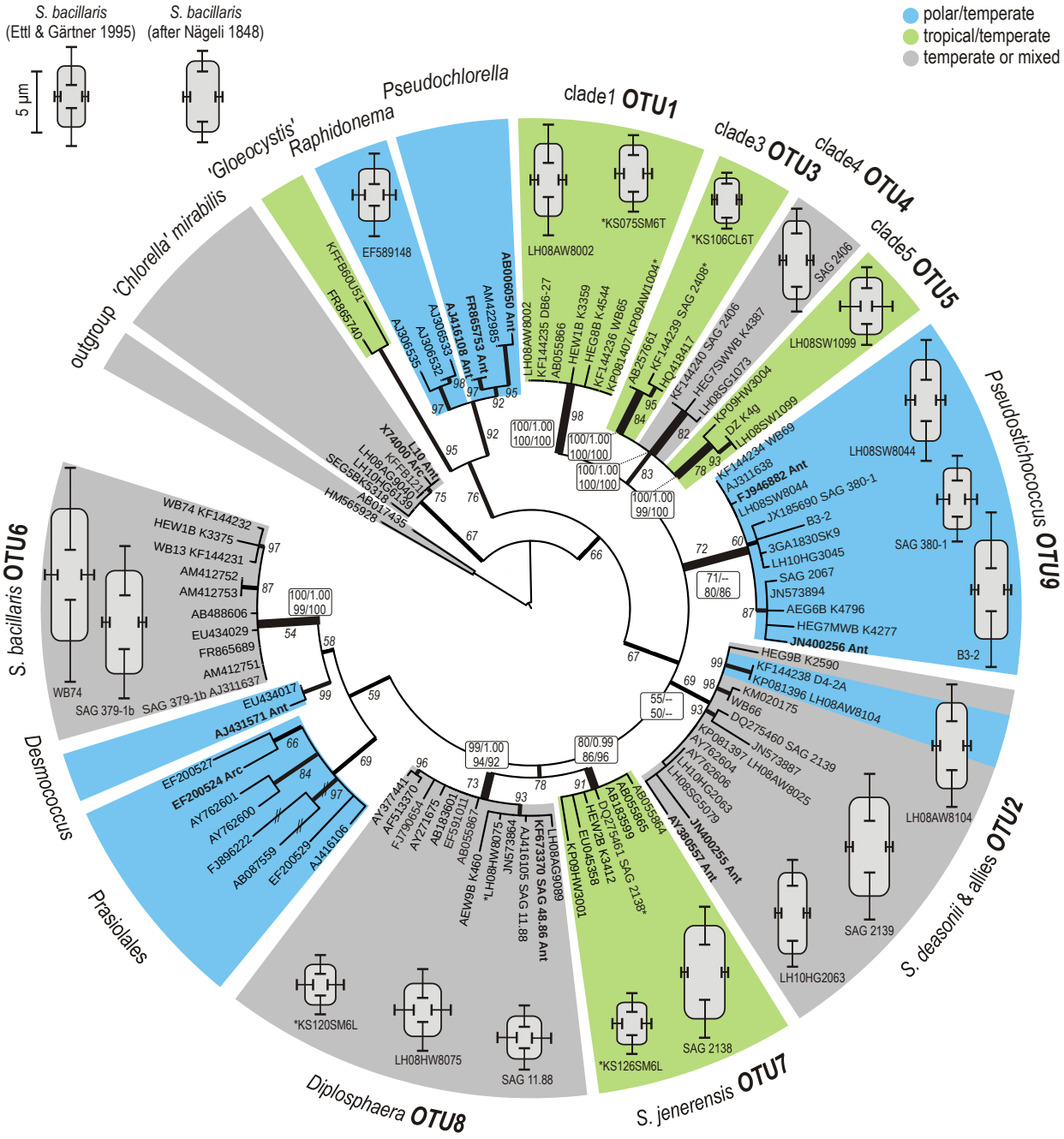

Figure 6. 18S ML (quartet puzzling) phylogeny of *Stichococcus*-like species and relatives. The numbers next to branches (italic letters) indicate bootstrap support values inferred from 1000 replicates. The numbers in white boxes close to clades studied in detail correspond to statistical values in the following order: ML/BI/MP/NJ. Schematic drawings within clades show cell size of representative *Stichococcus*-like strains as compared to *Stichococcus bacillaris* morphospecies after Nägeli (1849) and Ettl & Gärtner (1995). Assignations into operational taxonomic units (OTUs) are based on sequence similarities ≥ 99.5% (= OTU) or ≥ 99.0% (= *OTU*). The clades are colored according to geographic origin of the sequences: blue=polar/temperate, green=tropical/temperate, grey=only temperate or temperate/polar/tropical.

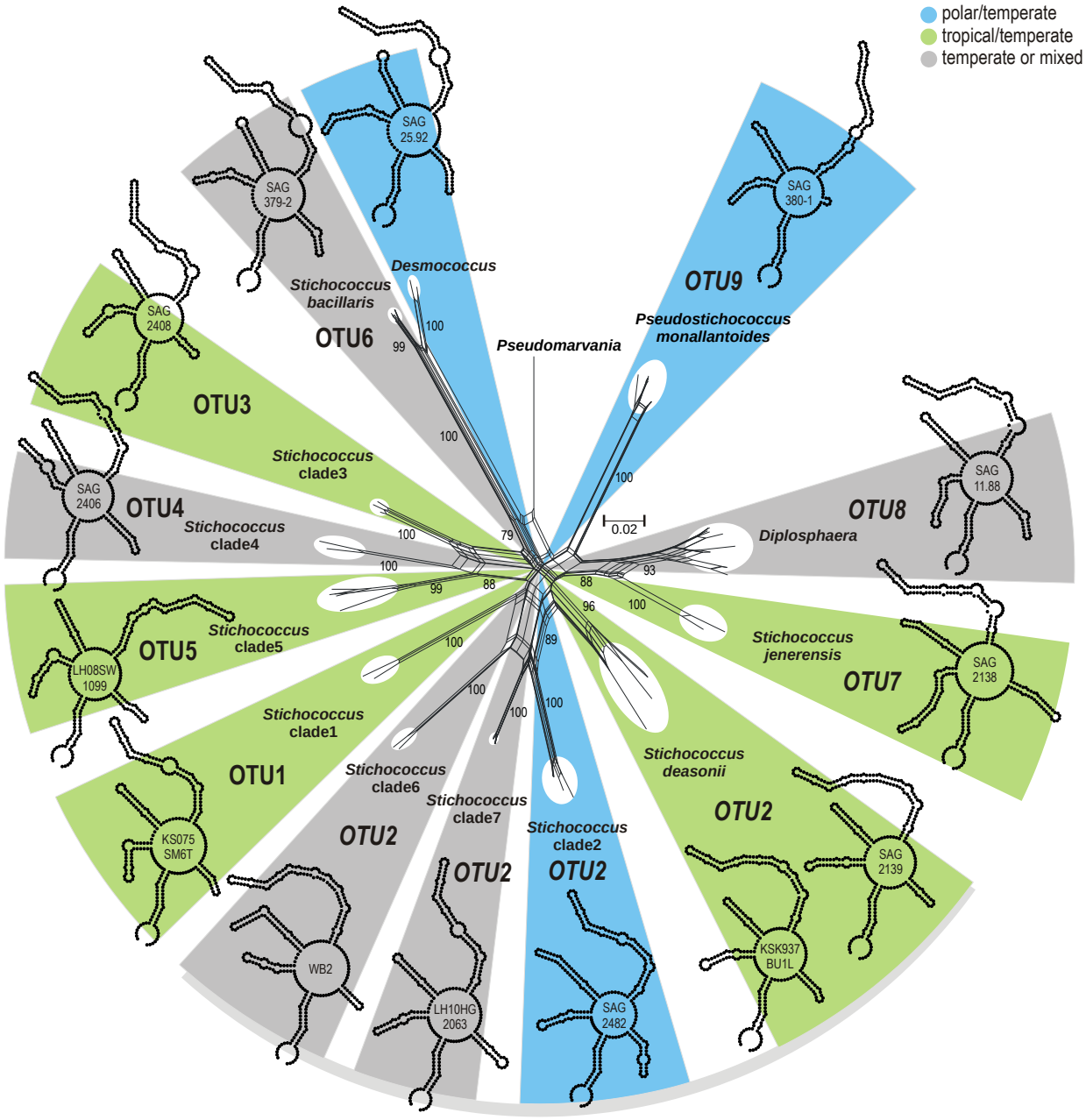

Figure 7. ITS2 phylogenetic network of *Stichococcus*-like species and relatives. Neighbor-net analysis of *Stichococcus* clades. The numbers near to splits correspond to bootstrap support values. Schematic ITS2 secondary structures are shown for representative strains. Assignations into operational taxonomic units (OTUs) are based on 18S inference (**Fig. 6**; **Table S2**). The comprehensive dataset is shown in **Fig. S5**. The clades are colored according to geographic origin of the sequences: blue=polar/temperate, green=tropical/temperate, grey=only temperate or temperate/polar/tropical.

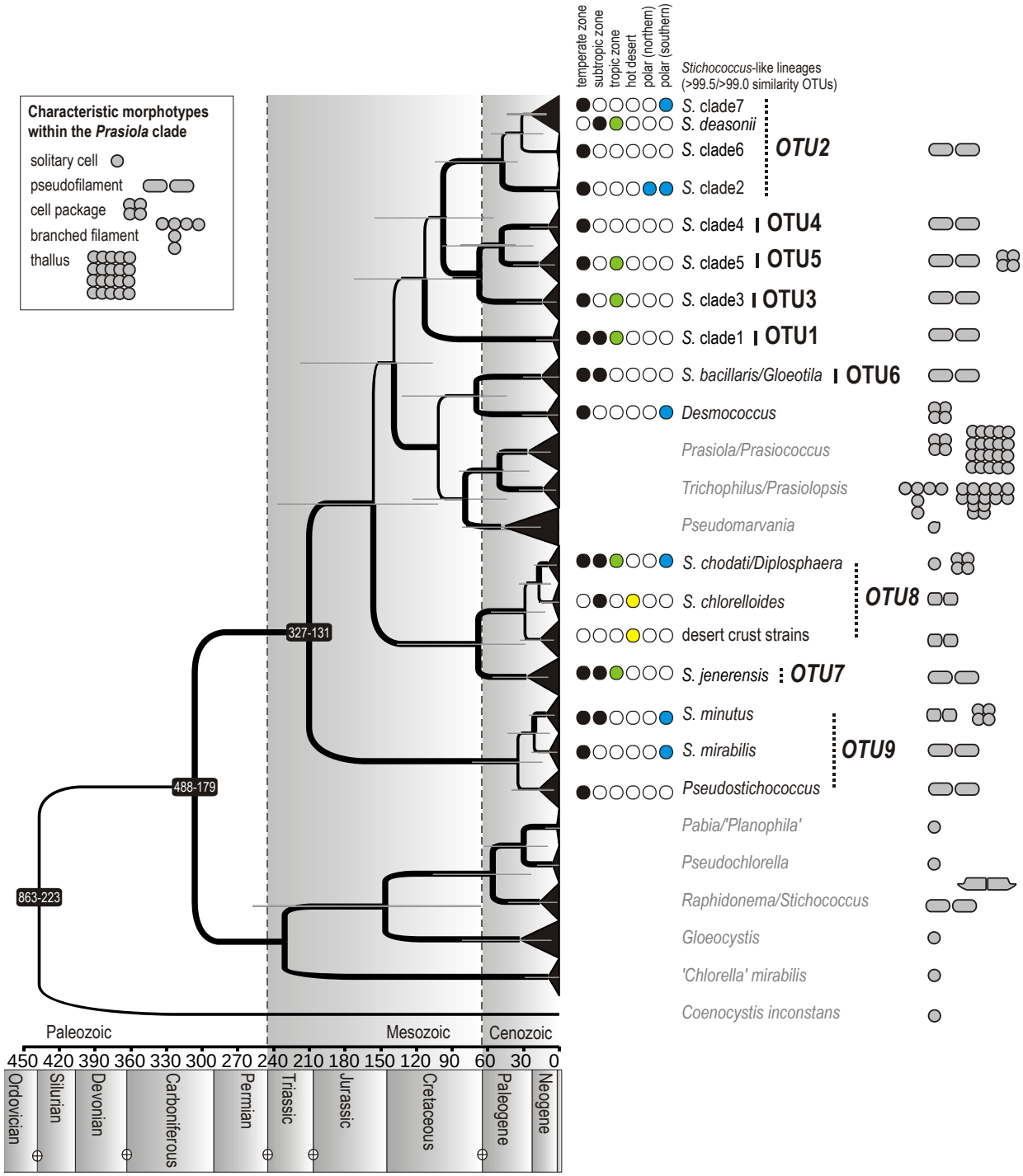

Figure 8. Calibrated 18S phylogeny of *Stichococcus*-like species and relatives using relaxed molecular clock. Bayesian phylogenetic tree (BEAST) computed from 18S dataset identical to **Fig. 6**. Colored dots to the right of the tree summarize information on the geographic distribution of *Stichococcus*-like species and relatives (based on molecular data listed in **Table S2**). Schematic drawings illustrate the multiple occurrence of rod-shaped (*Stichococcus*-like) morphotypes throughout the *Prasiola* clade. Assignations into operational taxonomic units are based on sequence similarities ≥ 99.5% (= OTU) and ≥ 99.0% (= *OTU*), **Fig. 6**.

Discussion

We defined operational taxonomic units (= clades consisting of identical or closely related sequences) as those with ≥ 99.5% SSU-sequence similarity. The most psychrotolerant strains of *Chlorella* and *Stichococcus* are conspecific (or closely related) with strains from the temperate zone. Including our new sequences and the sequences published in GenBank (De Wever *et al.* 2009), we recognized seven phylogenetic species of polar Chlorellaceae. Five of these species are the psychrotolerant Chlorellaceae isolated by Shukla *et al.* (2011). Such a low phylogenetic diversity of *Chlorella* is also known from arid regions in which six species have been reported so far (Lewis and Lewis 2005; Flechtner *et al.* 2013; Fučíková *et al.* 2014). Our results suggest that particularly two species of the Chlorellaceae, *Chlorella vulgaris* (Bock *et al.* 2011) and *Muriella terrestris* (Hanagata 1998), might be distributed in both polar and arid desert environments. Terrestrial *Stichococcus*-like microalgae might be even more widely dispersed than members of the Chlorellaceae, because in addition to the polar and temperate regions, they were detected in the tropics as well. However, six *Stichococcus* clades with a temperate-polar distribution do not include any tropical relatives. And vice versa, *Stichococcus* clades with a temperate-tropical distribution do not contain any psychrotolerant species.

Chlorella-like psychrotolerant strains have allies in temperate zones and hot deserts

The strains morphologically resembling *Muriella* (Petersen 1935) are phylogenetically nested in *Muriella terrestris* (Hanagata 1998). We detected a considerably low genetic distance–a difference of three nucleotides within ITS2–among the *M. terrestris* strains sampled in Antarctica (L20; Shukla *et al.* 2011; Kochkina *et al.* 2014) and Germany (LH08SG3009). Their putative conspecifity is supported by the absence of compensatory base changes (CBCs; Müller *et al.* 2007b; Wolf *et al.* 2013). Further, SSU-based evidence suggests a widespread distribution of *M. terrestris* in streams of high CO_2-pressure (Hodač *et al.* 2015), freshwaters (Fawley *et al.* 2004) and even in hot deserts (Flechtner *et al.* 2013; Fučíková *et al.* 2014). Another *Nannochloris*-like clade includes the Antarctic isolates L13/L14/L15/L23/L32 (Shukla *et al.* 2011), which are weakly related to *Marvania geminata* (Henley *et al.* 2004; Eliáš and Neustupa 2009). These strains cluster together with another Antarctic *Nannochloris* sp. Ant-1 (Gilichinsky *et al.* 2007), with *Chlorella* sp. 193-GA188 from the Siberian permafrost (Vishnivetskaya 2009) and with multiple isolates from German soils (e.g., LH08SG2053) and from freshwater (*Nannochloris* sp. JL 4-6; Fawley *et al.* 2004). The relatives of the tropical species *Hindakia fallax* (Bock *et al.* 2010) exhibited low genetic distance (three nucleotides in ITS2) between the isolate N9 from

Svalbard (Shukla *et al.* 2011) and the Antarctic isolate KNUA034 (Hong *et al.* 2015). The Arctic isolate N9 is phylogenetically nested in a clade containing other accessions provisionally denominated as *Chlorella* spp., a putative sister lineage to the tropical *Hindakia fallax*. *Hindakia* spp. was already recorded multiple times from Antarctica (Hu *et al.* 2008; De Wever *et al.* 2009; Hong *et al.* 2015) and includes freshwater strains, which were screened for application in biotechnology, e.g., CCAP 211/79 (Germond *et al.* 2013; Osundeko *et al.* 2013; Driver *et al.* 2015). High ITS2 similarity between isolates from Nova Scotia (KP204587; Park *et al.* 2015) and the South China Sea (GU942201), which are both relatives of the Antarctic isolate L3 (Shukla *et al.* 2011), additionally supports the long-distance dispersal of *Chlorella* spp.

Chlorella vulgaris, the epitome of spherical green microalgae (Krienitz *et al.* 2015), was isolated from Antarctica and represented by the intensively investigated psychrotolerant strain NJ-7 (Li *et al.* 2009; Lu *et al.* 2009; Lu *et al.* 2010). The SSU sequences of our Antarctic (L1/L4) and Arctic (L5/L6) isolates (Shukla *et al.* 2011) clustered between *C. vulgaris* SAG 211-11b and its closest named relative *C. pituita* ACOI 311 (Bock *et al.* 2011). The Antarctic strains are more different from the temperate *C. vulgaris* SAG 211-11b than the Arctic strains; the most similar strain to the Antarctic strains L1/L4 was isolated from a hot desert (JX446471; Flechtner *et al.* 2013) and a German soil (LH10HG2081; this study). ITS2 analysis of multiple *C. vulgaris* strains revealed considerable intraspecific diversity; the particular ITS2 ribotypes do not cluster according to their original habitat (freshwater vs. terrestrial). Remarkably, the ITS2 of the Antarctic strain L4 differs from all *C. vulgaris* strains, but is almost identical to the isolate LH10HG2081. Taking the ITS2 difference between *C. vulgaris* SAG 211-11b and *C. pituita* ACOI 311 (32 nucleotides and 3-4 CBCs) into account, then the psychrotolerant (L4) and *C. vulgaris* SAG 211-11b might be conspecifics (differing in 14 nucleotides; 0 CBC). In comparison, another trebouxiophycean genus *Coccomyxa* exhibits intraspecific variability of 26 nucleotide positions in ITS2 (Darienko *et al.* 2015). Evidence of identical ITS2 ribotypes from Europe (SAG 211-11b) and North America (CCAP 254/5) confirms the long-distance dispersal of *C. vulgaris*. *Chlorella sorokiniana* (Bock *et al.* 2011) is another remarkable *Chlorella*, so far known from both cold and hot environments, e.g., the Antarctic (De Wever *et al.* 2009) and North-American deserts (Flechtner *et al.* 2013). In contrast to the terrestrial *Chlorella*-like species mentioned above, *C. sorokiniana* might be dispersed even in the humid tropics and was recorded (based on DNA data) from Central America (de-Bashan *et al.* 2008), South America (de-Bashan *et al.* 2008; Bashan *et al.* 2015) and Southeast Asia (identified by S. Marimuthu; http://studentsrepo.um.edu.my/3562/). The species is thermotolerant (de-Bashan *et al.* 2008; Zheng *et al.* 2013). Other thermotolerant Chlorellaceae recorded from hot deserts have close

relatives from phytoplankton, e.g., *Meyerella* (Fučíková *et al.* 2014), *Micractinium* (Flechtner *et al.* 2013); the distribution of the recently described *Chlorella thermophila* (Ma *et al.* 2015) remains unknown. Apart from the psychrotolerant strains described above, another two polar strains, i.e., L24 (Shukla *et al.* 2011) and VPL1-3 (FJ946890; De Wever *et al.* 2009) are incertae sedis in the Chlorellaceae.

***Stichococcus*-like clades including psychrotolerant species and species from hot deserts**

The psychrotolerant strains of *Stichococcus* which have been sequenced so far (Hughes 2006; Chen *et al.* 2012) cluster within three different lineages of the *Stichococcus* clade sensu Sluiman and Guihal (2008). We provisionally denominated these clades as *Pseudostichococcus* (SAG 2067; SAG 380-1; CCAP 379/3), *Stichococcus* clade2 (SAG 2482) and *S.* clade7 (SAG 2060; SAG 2119). The *Pseudostichococcus* clade (including the strain *P. monallantoides* SAG 380-1; Moewus 1951) is an early diverging lineage consisting of species morphologically resembling *Stichococcus* (Nägeli 1849) and *Desmococcus* (Brandt and Stockmayer 1925; Gärtner and Ingolić 2003). Two Antarctic strains are nested within the *Pseudostichococcus* clade: Trebouxiophyceae EO7-4 (De Wever *et al.* 2009) and *Stichococcus minutus* NJ-17 (Chen *et al.* 2012). The freshwater strain EO7-4 is genetically similar to German isolates from soil (LH08SW8044; this study) and a karst-water stream (WB69; Hodač *et al.* 2015). The allies of the subaerial isolate NJ-17 were detected as environmental clones from German soils and isolated from a lichen (Thüs *et al.* 2011). Further Antarctic strains–phylogenetically distant from the *Pseudostichococcus* clade–were isolated from permafrost (*Stichococcus bacillaris* s3; Hughes 2006) and wet rocks (*S. bacillaris* NJ-10; Chen *et al.* 2012) and belong to a poorly resolved clade provisionally denominated as *Stichococcus* clade7. As a result of the SSU-based phylogeny, the species within the *Stichococcus* clade7 might be allies of *S. deasonii* (Neustupa *et al.* 2007). The ITS2-based inference, however, did not unambiguously support the monophyletic origin of both clades, suggesting a considerable evolutionary distance between *S.* clade7 (including the above mentioned polar species) and the *S. deasonii* lineage (including tropical species). Notably, the *Stichococcus* clade7 further consists of species from soils and subaerial habitats (Karsten *et al.* 2005), but, in contrast to most *Stichococcus*-like clades, no aquatic species are known so far. The only psychrotolerant *Stichococcus*-like strain obtained from the Arctic permafrost is *Stichococcus* sp. 594-GA18 (Vishnivetskaya 2009) isolated from a 4.65 m depth in Siberia. This strain clusters within the *Stichococcus* clade2, an unnamed monophyletic lineage closely related to the above mentioned *S.* clade7. *Stichococcus* clade7 contains German isolates from a karst-water stream (D4-2A; Hodač *et al.* 2015) and soils (e.g., SAG 2482). Remarkably, *S.* clade7 is the only known

Stichococcus-like clade which might have a bipolar distribution; it includes environmental clones (HM490287, HM490288; Khan *et al.* 2011) from the extreme McMurdo Dry Valleys, Antarctica, which are highly similar to the above mentioned soil strain SAG 2482 (6-9 nucleotide differences in ITS2, no CBCs).

The phylodiversity of Chlorellaceae in hot deserts might be higher than that of *Stichococcus*, which is limited to a single known clade. This clade including desert species we denominated *Diplosphaera*, based on the species *D. mucosa* (Broady 1983) and *D. sphaerica* (Karbovska and Kostikov 2012b). *Diplosphaera mucosa* SAG 48.86 is the only polar strain within the *Diplosphaera* clade, however, the clade contains another putatively psychrotolerant species, represented by the environmental clone QE28 detected in the Tibetan tundra (FJ790654; Wong and Lacap 2010). The members of the *Diplosphaera* clade inhabit not only extremely cold and hot deserts (Lewis and Lewis 2005; Flechtner *et al.* 2013), but also acidic freshwater (Aguilera *et al.* 2007), oceanic sediment (AB183601) and occur as common lichen photobionts (Thüs *et al.* 2011; Fontaine *et al.* 2012; Fontaine *et al.* 2013). *Diplosphaera* ITS2 sequences, which were retrieved from leaf surfaces in an Ecuadorian tropical rainforest (e.g., KS120SM6L), were almost identical (i.e., differing in only three nucleotides; no CBC) to the isolate LH08HW8075 (this study) from a German forest soil. The SSU data suggest close relatedness of both temperate-tropical strains, the Antarctic *Diplosphaera mucosa* SAG 48.86 and lichen photobiont *Diplosphaera* sp. J4028B (Thüs *et al.* 2011). In *Stichococcus* clade7, we also observed genetic relatedness of a lichen photobiont (*Diplosphaera* sp. W1118; Thüs *et al.* 2011), tropical clone KSK870SM6T and polar isolates (e.g., *Stichococcus bacillaris* NJ-10; Chen *et al.* 2012), however, this evidence is based on lower genetic similarities as compared to the *Diplosphaera* clade.

Biogeography of terrestrial *Chlorella* and *Stichococcus*

Patterns in species distribution have been accepted for freshwater microalgae (De Wever *et al.* 2009; Naselli-Flores and Padisák 2015). Some freshwater species of "true" *Chlorella*, which were described from the tropics (e.g., *C. pulchelloides, C. rotunda, C. singularis, C. volutis*; Bock *et al.* 2011), have not yet been uncovered in polar regions. Furthermore, the *Chlorella*-like species we detected in Antarctica and the Arctic (*C. vulgaris*, *C.* spp., *Muriella terrestris*, *Marvania*-relative) belong to clades with temperate-polar distributions; they are genetically divergent from their tropical relatives (e.g., *Hindakia fallax* or *Chlorella sorokiniana*). However, concerning terrestrial green microalgae, Earth´s biodiversity hotspots such as tropical rainforests still remain largely unexplored. Morphology-based observations suggest that true *Chlorella* (and other

Chlorellaceae) might be less common in tropical rainforests than *Stichococcus* (Neustupa and Škaloud 2008; 2010). Accordingly, the most *Chlorella*-like species, which were described from tropical rainforests using molecular data, belong either to the trebouxiophycean *Watanabea* clade (Zhang *et al.* 2008; Neustupa *et al.* 2009; Suutari *et al.* 2010; Song *et al.* 2015) or to the class Chlorophyceae (e.g., *Jenufa*; Němcová *et al.* 2011; Hodač *et al.* 2015). Preliminary cloning studies (unpublished data of the coauthors of this study) on the diversity of terrestrial green microalgae in Ecuadorian rainforests revealed only *Chlorella* sequences related to *C. sorokiniana*, a putatively pantropic and thermotolerant species. Remarkably, common terrestrial species such as *Chlorella vulgaris* or *Muriella terrestris* were not detected, although the approach did uncover common species of *Stichococcus*, which were almost identical to isolates from European soils and freshwaters. The *Stichococcus* species with temperate-tropical distribution were provisionally denominated as *Stichococcus* clade1, *S.* clade3 and *S.* clade5, and do not contain any psychrotolerant strains. The same is true for the first *Stichococcus* described from the tropics, *S. jenerensis* SAG 2138 (Southeast Asia; Neustupa *et al.* 2007). The discovery of *S. jenerensis* as well in Ecuador (KS126SM6L) confirmed the pantropic-temperate distribution of the *S. jenerensis* clade.

The deepest divergence within the *Stichococcus* clade (Sluiman and Guihal 2008), or *Stichococcus/Prasiola* clade (Neustupa *et al.* 2007) occurred in the Mesozoic, during the transition from the Lower Jurassic to Middle Cretaceous (De Wever *et al.* 2009). According to the same study, the deepest splits within the Chlorellaceae would be dated much earlier, i.e., approximately in the Lower Devonian to Lower Jurassic. The earliest divergence of the Antarctic versus non-Antarctic species within the *Chlorella* clade would thus have happened during the Cenozoic (Upper Paleocene to Upper Oligocene). Our accordingly calibrated phylogeny suggests a remarkable coincidence of (1) species divergence in the *Chlorella* clade and (2) species divergence in the *Stichococcus* clade. During the same period in the Triassic, the following splits resulting in new lineages might have appeared: split 1) *Stichococcus jenerensis* (rod-shapes; temperate-pantropic distribution) and *Diplosphera* (rod-shapes/cell packages; temperate-tropic-arid-polar distribution); split 2) *Stichococcus bacillaris* (rod-shapes; temperate distribution) and *Desmococcus* (cell packages/filaments; temperate-polar distribution); split 3) *Stichococcus* clade3 (rod-shapes; temperate-tropical distribution) and *S.* clade4+clade5 (rod-shapes/cell packages; temperate-tropical-polar(?) distribution). In summary, *Stichococcus*- and *Diplosphaera/Desmococcus*-like morphologies evolved multiple times within the *Stichococcus* clade, possibly in successive order. The morphological plasticity of the *Stichococcus* lineages could explain its success in colonizing almost all terrestrial substrates (soil, epiphytic, epixylic,

epilithic, endolithic, lichens). In contrast to *Stichococcus*, the lineages of terrestrial Chlorellaceae are morphologically uniform, without any signs of multicellularity. The strikingly poor phylogenetic diversity of the Chlorellaceae found in humid tropical rainforests might be, apart from a simple sampling effect, due to low competitiveness. Instead, Chlorellaceae successfully colonized low-competitive microbial communities of extreme cold and hot terrestrial environments. Nonetheless, most regions on Earth have not been sampled yet. Although polar research undoubtedly contributes valuable biodiversity data, the picture of algal biogeography should be completed by much more samplings in rapidly disappearing biodiversity hotspots such as tropical rainforests.

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Chapter 4

Molecular evidence for the wide distribution of two lineages of terrestrial green algae (Chlorophyta) from tropics to temperate zone

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Abstract

Phylogenetic analyses of 18S rDNA sequences from environmental clones and culture strains revealed a widespread distribution of two subaerial green algal lineages, *Jenufa* and *Xylochloris*, recently described from rainforests in Southeast Asia. A new lineage of *Jenufa* (Chlorophyceae), most closely related to or even conspecific with *J. minuta*, was formed by sequences of European origin. Two more lineages of *Jenufa* were formed by three additional sequences from Ecuador and Panama. The other lineage was a close relative of *Xylochloris irregularis* (Trebouxiophyceae), probably representing a new species of the genus and distinct from the only so far described species, *X. irregularis*. It comprised two distinct clades each containing almost identical sequences from Germany and Ecuador. Analyses of the new sequences for both genera allowed to presume a preference of *J. minuta* to subaerial growth on rock or artificial hard substrates combined with a remarkable adaptation to extended periods of darkness, whereas *Xylochloris* may preferably occur on tree bark or in the soil.

Keywords: green algae; *Jenufa*; *Xylochloris*; molecular phylogeny; 18S rDNA; biogeography; substrate specificity

Abbreviations: BioNJ, Bio-neighbor-joining; CS, chlorophyte superclades including Chlamydomonadales and Sphaeropleales; MB, Bayesian inference; ML, Maximum likelihood; MP, Maximum parsimony; NMDS, Non-metric multidimensional scaling

Introduction

The neutral dispersal model (Fenchel and Finlay 2004) suggests that microorganisms do not exhibit biogeography, i.e. they are so small that no distributions barriers exist for them. This has been subject of debates by several authors, i.e. geographic distribution was found in ciliates (Foissner *et al.* 2008) and some microalgae (Řezáčová and Neustupa 2007). A recent study based on 18S rDNA sequence comparisons provided evidence for endemism in an Antarctic habitat, i.e. several new independent lineages of green algae were found in the benthos of an Antarctic lake (Lawley *et al.* 2004; De Wever *et al.* 2009). However, caution needs to be applied because there may still be an insufficient number of sequences available for many groups within the green algae. A lineage may appear "endemic" only as long as no other close relatives to it are known and this may easily change with the availability of new sequences from close relatives. In contrast to benthic freshwater algae, subaerial algae may be widely dispersed due to their remarkable adaptation to fast changing and adverse environmental conditions. Their resting cysts may easily survive transportation over long distances in the air being resistant to drought, high as well as low light intensities, and high UV radiation, e.g. due to the presence of thickened cell walls or light protection pigments (Řezanka *et al.* 2004; Karsten *et al.* 2005; Häubner *et al.* 2006; Gustavs *et al.* 2010).

Recently, subaerial microalgae from Southeast Asian tropical rainforest habitats gained increased interest by a series of studies (Eliáš *et al.* 2008; Neustupa *et al.* 2009; Eliáš *et al.* 2010; Němcová *et al.* 2011; Neustupa *et al.* 2011) which lead to the establishment of two new genera of green algae, *Jenufa* (Němcová *et al.* 2011) and *Xylochloris* (Neustupa *et al.* 2011). Both genera appeared as somehow distant to any known genera of green algae, despite a taxon sampling as large as possible has been applied in the 18S rDNA sequence analyses of both studies. The environmental sequencing approach extends the data pool available for diversity comparisons and may enable us to assess distribution patterns of protists (Šlapeta *et al.* 2005; Edgcomb *et al.* 2011). In the present study we took the advantage of an extended taxon sampling available through new 18S rDNA environmental sequences we obtained from our own ongoing studies which focus on changes in the algal diversity of certain terrestrial habitats along gradients of abiotic parameters in Europe (Germany, Ukraine) and South America (Ecuador). In addition, a variety of closely related environmental sequences have become available from GenBank. Here we demonstrate the presence of new lineages for both recently described genera, *Jenufa* (Chlorophyceae) and *Xylochloris* (Trebouxiophyceae), their wide distribution over long geographic distances and that both differ in their preferences towards certain substrates.

Materials and methods

Origin of analyzed sequences

The analyzed sequences and culture strains were provided by several our own on-going studies which focus on the algal diversities of various terrestrial habitats, see **Table 1**. They were derived from soil, tree bark or stone surfaces samples at four temperate habitats and one tropical habitat (**Table 1**). The majority of sequences were from environmental 18S rDNA clone libraries (334-1761 base pairs long), three from cultured strains which were accessioned by the SAG culture collection as strains SAG 2379, SAG 2382, and SAG 2383. Soil and tree bark samples originating from Germany were collected within the frame of the German Biodiversity Exploratories project (http://www.biodiversity-exploratories.de), for project description see Fischer *et al.* (2010) (**Fig. 1a**). Samples of biofilms dominated by green algae were investigated from the surface of sandstone samples within the framework of another study, focusing on wall sections of the castle "Burg Gleichen", near Gotha (Thuringia, Germany) (Hallmann *et al.* 2011a). The biofilm samples were either from a sun-exposed wall area (**Fig. 1b**) or from the inner face of a scale from a dark basement vault (**Fig. 1c**). Samples from an even less light-exposed locality were from the concrete walls inside a World War II bunker monument on the North Sea offshore island Helgoland, Germany (**Fig. 1d-e**). Here the algal biofilms were exposed to extended periods of darkness because light is available from fluorescent tubes only during guided tours for tourists, i.e., 1.5 hours per day and around 8 months per years. The soil sample from Ukraine was from the arid steppe zone (Chernomorskiy Biosphere Reserve). The soil and tree bark samples from South America originated from the tropical mountain rain forest in Southern Ecuador along an elevation transect at 1000 m, 2000 m and 3000 m above sea level (a.s.l.) corresponding to three different regions (**Table 1**; **Fig. 1f-g**).

Microscopy and rDNA sequence determination

Microscopic observations of cultures were conducted using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics and an attached ColorView III camera (Soft Imaging System, Münster, Germany). Micrographs were processed using Cell^D image software (Soft Imaging System, Münster, Germany). The detailed experimental procedures on how the 18S rDNA clone libraries and cultures were established will be described elsewhere. Briefly, DNA was extracted from environmental samples using MoBio PowerSoil DNA isolation Kit (MoBio Laboratories Inc. Carlsbad, CA, USA) and from mechanically disrupted cultured algal cells using Invisorb® Spin Plant Mini Kit (Stratec, Berlin, Germany). Almost full length 18S rDNA

sequences were amplified from the cultures and European environmental samples with primers preferentially binding to green algal rDNAs, for the samples from Ecuador a region from about 350 base pairs upstream of the 3' end of 18S rDNA downstream to the 26S rDNA which included the ITS2 rDNA for DNA barcoding purposes was amplified. Either almost full 18S rDNA sequences were obtained or partial sequences comprising either the hypervariable regions V2, V3 and V4 (European environmental samples) or hypervariable regions V8 and V9 regions (Ecuador environmental samples) of 18S rDNA (Neefs and De Wachter 1990; Lee and Gutell 2012). The newly determined sequences are available from GenBank under the accession numbers JQ988923-JQ988943.

Table 1. List of new sequences obtained for this study.

Taxon	Clone identifier (*cultured strains)	Accession number	Length (base pairs)	Region of origin	GPS-coordinates	Habitat	Substrate
Xylochloris	AEG1B-K1547	JQ988937	748	Germany, Swabian Alb	48°23'N, 9°20'E	Grassland	Soil
	AEW2R-K255	JQ988938	1734	Germany, Swabian Alb	48°37'N, 9°35'E	Spruce forest	Tree bark, *Picea abies*
	AEW2R-K265	JQ988934	842	Germany, Swabian Alb	48°37'N, 9°35'E	Spruce forest	Tree bark, *Picea abies*
	BOAEW3R1-K03	JQ988939	716	Germany, Swabian Alb	48°41'N, 9°35'E	Spruce forest	Tree bark, *Picea abies*
	SAG 2382*	JQ988942	1719	Germany, Swabian Alb	48°39'N, 9°26'E	Beech forest	Soil
	HEG9B-K2617	JQ988940	1702	Germany, Hainich	51°13'N, 10°22'E	Grassland	Soil
	HEW10SB-K5621	JQ988935	854	Germany, Hainich	51°05'N, 10°27'E	Beech forest	Soil
	HEW4SB-K5577	JQ988936	854	Germany, Hainich	51°24'N, 10°32'E	Beech forest	Soil
	HEW4SB-K5606	JQ988941	733	Germany, Hainich	51°24'N, 10°32'E	Beech forest	Soil
	U33B-K2708	JQ988943	628	Ukraine, Ascania Nova	46°27'N, 33°54'E	Steppe	Soil
	C32U6-13	JQ988925	334	Ecuador, Cajanuma	04°06'S, 079°10'W	Rainforest 3000 m a.s.l.	Soil
	S30L6-32	JQ988926	334	Ecuador, San Francisco	03°58'S, 079°04'W	Rainforest 2000 m a.s.l.	Tree bark
Jenufa	3GA1K3658	JQ988931	851	Germany, Drei Gleichen	50°52'N, 10°50'E	Castle Gleichen	Sandstone, surface
	GS1K32	JQ988932	1761	Germany, Drei Gleichen	50°52'N, 10°50'E	Castle Gleichen	Sandstone, scale (endolithic)
	KB2B11-06	JQ988928	1596	Germany, Helgoland	54°11'N, 7°53'E	Bunker	Concrete
	KB4B5-06	JQ988929	1570	Germany, Helgoland	54°11'N, 7°53'E	Bunker	Concrete
	SAG 2379*	JQ988927	1563	Germany, Helgoland	54°11'N, 7°53'E	Bunker	Concrete
	SAG 2383*	JQ988933	1700	Germany, Swabian Alb	48°38'N, 9°38'E	Beech forest	Soil
	FLUL-B69M5-34	JQ988923	335	Ecuador, Bombuscaro	04°07'S, 078°58'W	Rainforest 1000 m a.s.l.	Tree bark
	S46L1-7	JQ988924	338	Ecuador, San Francisco	03°58'S, 079°04'W	Rainforest 2000 m a.s.l.	Soil

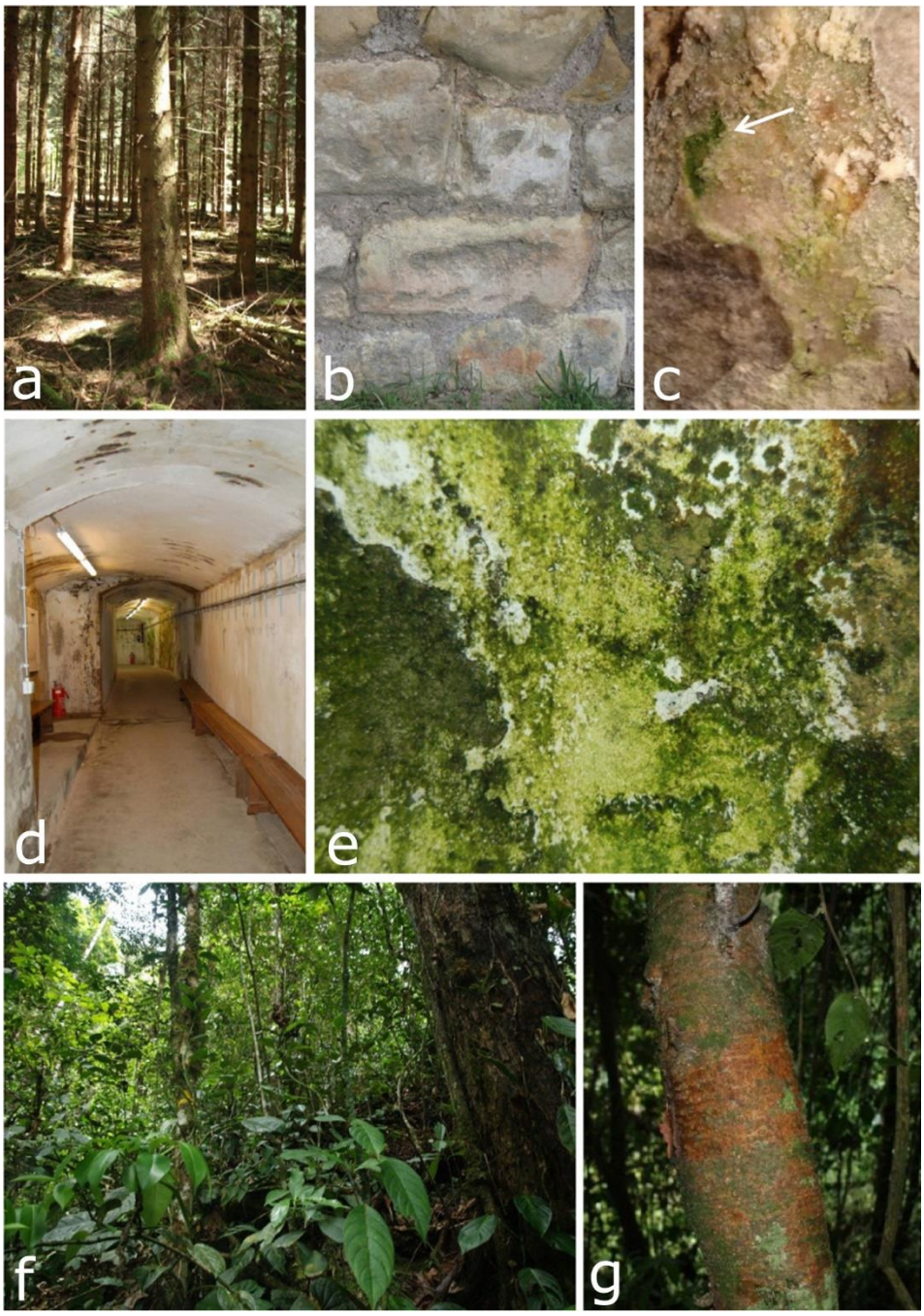

Figure 1. Sampling sites. (a) Young spruce forest in the Swabian Alb, (b-c) wall sections of castle Gleichen (Germany), (b) sun-exposed wall area, (c) inner face of a scale from a dark basement vault (the white arrow shows the sampled green biofilm), (d-e) biofilm samples from concrete walls in a World War II bunker on the North Sea offshore island Helgoland, (e) sampled green biofilm in detail, (f-g) tropical sub-mountain rainforest in Ecuador (the study site in Bombuscaro, 1000 m a.s.l.), and (g) green biofilms on tree bark in detail.

Phylogenetic analyses

The newly determined sequences were used in database queries using BLAST (Altschul *et al.* 1997) at NCBI (http://www.ncbi.nlm.nih.gov/) to retrieve their closest neighboring sequences available from public sequence data bases (state in March 2012). In addition, the newly determined and their retrieved closest relative sequences were compared to a broad selection of green algal sequences which was available in the internal 18S rDNA sequence database of our laboratory and is maintained in the ARB program (version 05.05.26, 2004, www.arb-home.de) (Ludwig *et al.* 2004). This database was updated with all currently available 18S rDNA sequences from green algae. The comparisons identified sequences of both genera, *Jenufa* and *Xylochloris*, as well as their next relatives. Potential chimeras were identified by Bellerophon (Huber *et al.* 2004) and excluded from the analyses. The almost full 18S rDNA sequences of *Jenufa*, *Xylochloris* and their close relatives were aligned in two separated data sets, i.e. together with representatives of all known lineages from the two green algal classes, Chlorophyceae and Trebouxiophyceae. First the sequences were aligned with MAFFT ver. 6 (Katoh and Toh 2008) online (http://mafft.cbrc.jp/alignment/server/index.html) in order to identify and exclude intron positions. Then the sequences from each class were aligned with MUSCLE online (http://www.ebi.ac.uk/Tools/msa/muscle). The alignments were carefully checked for possible misaligned positions by eye in BioEdit (Hall 1999). The final alignment of Chlorophyceae comprised 97 sequences and 1790 positions (742 variable/535 parsimony informative sites), the alignment of Trebouxiophyceae included 99 sequences and 1807 positions (710 variable/497 parsimony informative sites). Based on the AIC criterion in jModelTest 0.1.1 (Posada 2008), the GTR+Γ+I nucleotide substitution model was selected as to fit best both alignments. ML phylogenies were obtained from RAxML 7.0.4 (Stamatakis *et al.* 2008). Confidence values for the obtained groups (edge support) were inferred from rapid bootstrapping algorithm (1000 replicates) as implemented in RAxML. Bayesian posterior probabilities for internal nodes were obtained using MrBayes 3.2 (Ronquist *et al.* 2012). Two MCMC runs for four million generations each with one cold and three heated chains using the GTR+Γ+I+COV evolutionary model (parameters were estimated from the data) were performed with trees sampled every 100 generations. For comparisons of European with Ecuadorian environmental clones for which different regions of the 18S rDNA were determined (see above), only the hypervariable regions V8/V9 of the 18S rDNA could be used because they represent an overlapping sequence region available in both data sets (European data set comprised the full sequences whereas the Ecuadorian one the partial ones). Then only sequences of closest relatives were used for the

phylogenetic analyses, i.e. the investigated datasets comprised less than 10 sequences and were only about 300 base pairs long. Bio-Neighbor-Joining distance phylogenies based on Jukes-Cantor genetic distances and 1000 bootstrap replicates were computed in the program SeaView ver. 4 (Gouy *et al.* 2010). The parsimony analysis was conducted using DNAPARS as implemented in SeaView with 10 times randomization of the sequence order and 1000 bootstrap replicates. Evolutionary distances among selected sequences were computed as p-distances in MEGA5 (Tamura *et al.* 2011) with ambiguous alignment positions removed for each sequence pair.

Multivariate statistical analysis

In order to compare the clones originating from the different substrates within Europe (tree bark, soil, stone surfaces) Non-metric Multidimensional Scaling (NMDS) analysis based on genetic distances was computed. The analysis was conducted on the matrix of Jukes-Cantor genetic distances (within alignments comprising hypervariable regions V2, V3 and V4 of the 18S rDNA) as implemented in the program package PAST ver. 2.12 (Hammer *et al.* 2001). The reliability of the resulting ordination was examined by checking the stress values from Shepard-plot. The alignment comprising the new sequences of *Jenufa minuta* relatives included 8 sequences and 658 positions (**Fig. 2d**), whereas the other comprising the new sequences of *Xylochloris*-relatives included 10 sequences and 595 positions (**Fig. 3d**).

Results and Discussion

Jenufa lineage

The 18S rDNA phylogeny revealed the position of several of the obtained new environmental sequences and the three cultured isolates into the immediate vicinity of the genera *Jenufa* (Chlorophyceae, **Fig. 2a, 2b, 2d; Fig. 1 in Appendix**) and *Xylochloris* (Trebouxiophyceae, **Fig. 3a, 3b, 3d; Fig. 2 in Appendix**). We recovered sequences assigned to *Jenufa* from environmental clone libraries from Germany (soil, hard substrates), Ukraine (soil) and Ecuador (soil, tree bark) (**Table 1**). The full European environmental sequences and the culture strains formed a well-supported monophyletic cluster (p-distances 0.001-0.004) closely related to *Jenufa minuta*. The p-distances among European sequences and *J. minuta* (0.016 – 0.019) were about two times lower than between *J. minuta* and *J. perforata* (0.038). The partial environmental sequences from Ecuador constitute a lineage by its own positioned between *J. minuta* and *J. perforata*, but without significant bootstrap support (**Fig. 2d**). As already discussed previously in Němcová *et al.* (2011),

Jenufa may represent a distinct probably new lineage of Chlorophyceae of still unresolved relationship to the orders Sphaeropleales and Chlamydomonadales. Within the 18S rDNA phylogeny presented here, *Jenufa* is placed together with *Golenkinia* on the very basis of Chlamydomonadales, although without statistical support (ML/MB: 69/0.83 in **Fig. 2a**; **Fig. 1 in supplement**). In contrast, in Bayesian tree topology, *Jenufa*- and *Golenkinia* clades formed a lineage independent of the Chlamydomonadales and Sphaeropleales. The light-microscopic observations on the two available culture strains SAG 2383 and SAG 2379 were in agreement with their assignment to *Jenufa* inferred from the 18S rDNA phylogenetic analyses. Both strains exhibited morphological characters as previously described for *J. minuta* and *J. perforata* (**Fig. 4a**). Bot species were found indistinguishable from each other without the use of a confocal microscope and the analysis of cell wall structures (Němcová *et al.* 2011).

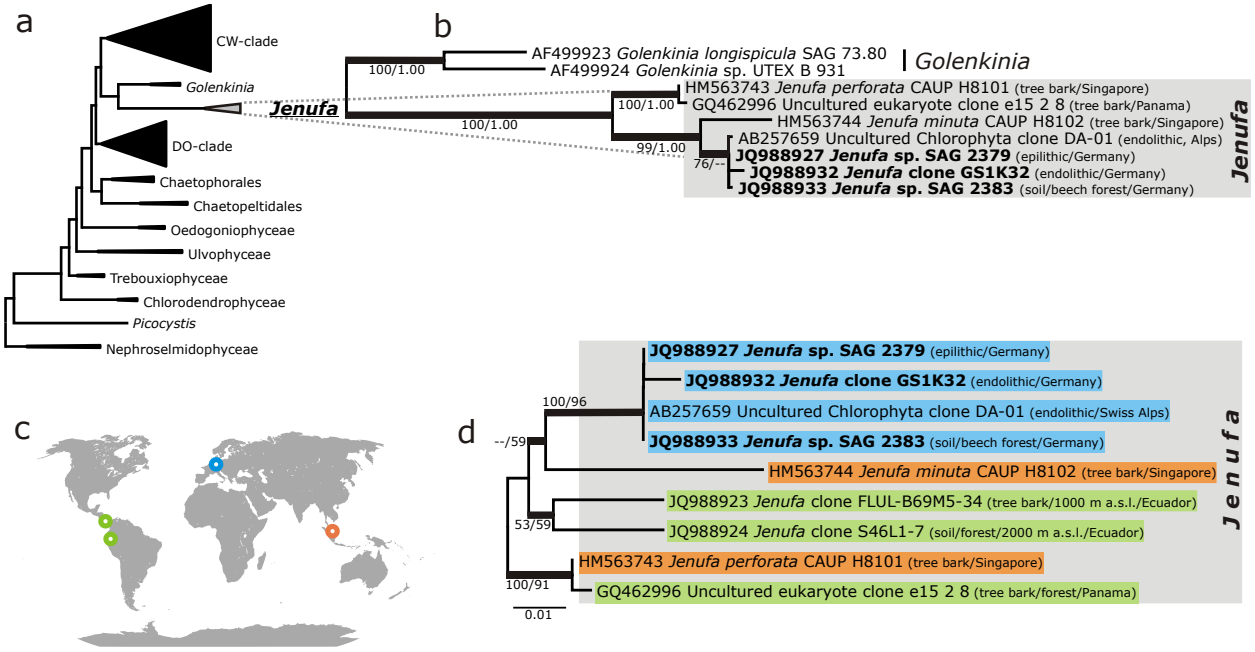

Figure 2. (a) Schematic 18S-based phylogenetic tree of the Chlorophyceae showing the position of sequences assigned to *Jenufa minuta*, (b) *Jenufa/Golenkinia* clades in detail shown as a section of the full 18S rDNA ML-phylogenetic tree (**Fig. S1 in Appendix**), (c) world map with dots representing regions where *Jenufa* sequences were detected, and (d) BioNJ-tree based on partial 18S sequences (hypervariable regions V8 and V9). Colors in figures (c) and (d) indicate three geographical regions where *Jenufa* occurs (orange—southeast Asia, green—south and Latin America, blue—Europe). Names in bold represent specimens analyzed as both partial- and full-length 18S rDNA sequences.

Xylochloris lineage

Other new environmental sequences and the sequence of strain SAG 2382 were closely neighboring to *Xylochloris irregularis* (Trebouxiophyceae) in the 18S rDNA phylogenetic analyses, but without significant bootstrap support (**Fig. 3b**). The p-distances among the full European sequences and *X. irregularis* (0.031 – 0.035) was slightly smaller than those between

the two previously described species of *Jenufa* (Chlorophyceae) which may imply that the European sequences and SAG 2382 represent a species independent of *X. irregularis*. The *Xylochloris* clade comprised environmental clones from soils and tree bark from Germany, Singapore and Ecuador (**Fig. 3d**). The addition of new sequences supported a sister-group relationship of *Xylochloris* with a clade comprising of environmental clones from cold-fumarole soils of the Andes (Costello *et al.* 2009) and two culture strains from terrestrial habitats in Germany assigned to *Dictyochloropsis* (**Fig. 3b, Fig. 2 in Appendix**). The latter clade was well supported in bootstrap tests, whereas its sister-group relationship with *Xylochloris* was only supported in MB analyses. Strain SAG 2382 shared several morphological characters with *X. irregularis* (Neustupa *et al.* 2011), i.e. the presence of a pyrenoid, the lobed chloroplast in vegetative cells, similar cell size, and the irregular cell shape. However, the strain also exhibited distinguishing characters of its own, namely the presence cylindrical as well as spherical autospores at the same time (**Fig. 4b-d**).

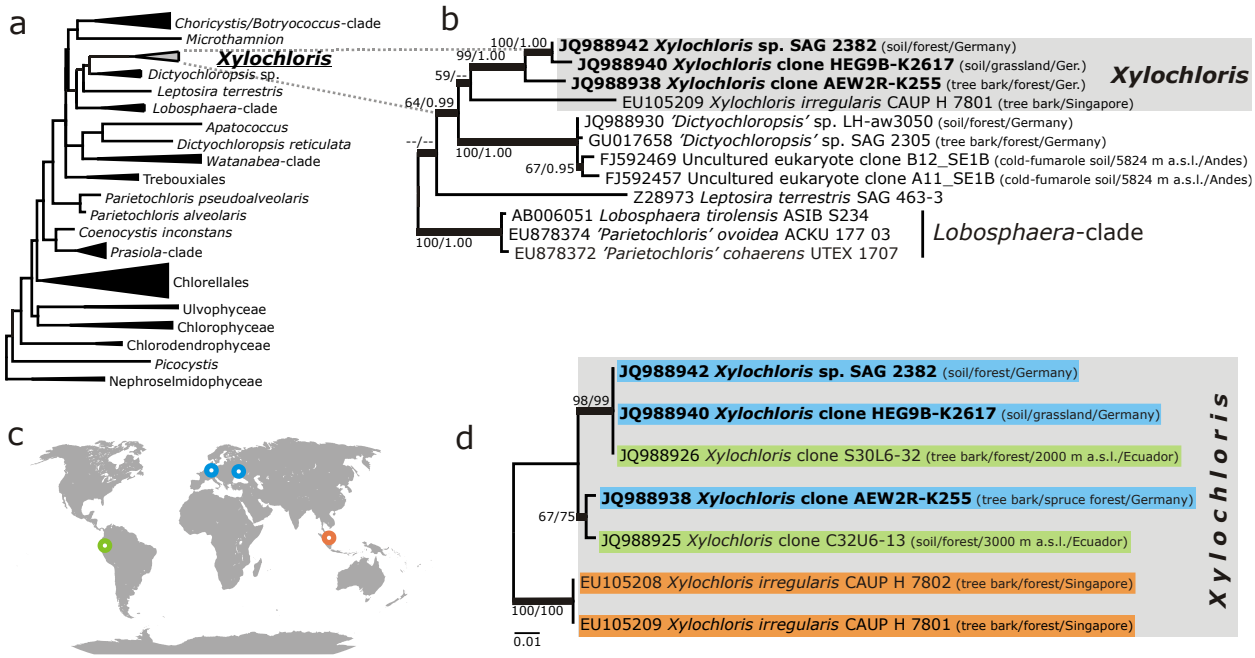

Figure 3. (a) Schematic 18S-based phylogenetic tree of the Trebouxiophyceae showing the placement of sequences assigned to *Xylochloris*, (b) the clade including *Xylochloris irregularis* and its closest relatives from Europe shown as a section of the full 18S rDNA ML-phylogenetic tree of the Trebouxiophyceae (**Fig. S2**), (c) world map with dots representing regions where *Xylochloris* sequences were detected, and (d) BioNJ-tree based on partial 18S sequences (hypervariable regions V8 and V9). Colors in Figures (c) and (d) indicate three geographical regions where *Xylochloris* occurs (orange—southeast Asia, green—South and Latin America, blue—Europe). Names in bold represent specimens analyzed as both partial- and full-length 18S rDNA sequences.

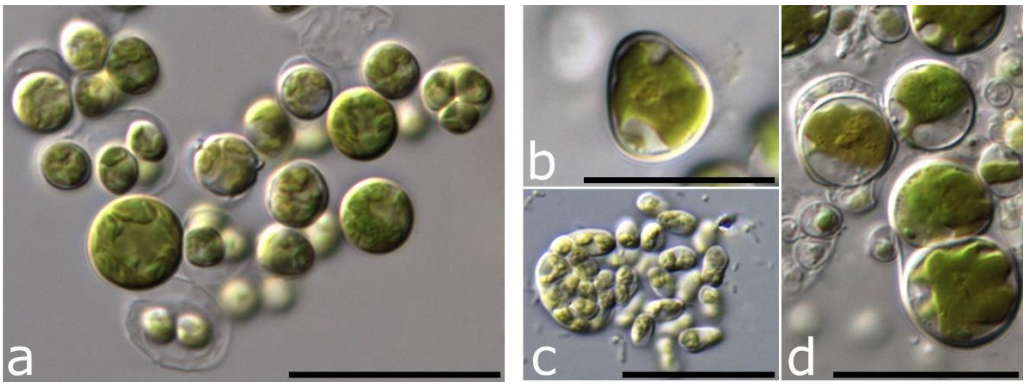

Figure 4. Microphotographs of the strains SAG 2383 (a) and SAG 2382 (b–d), (a) vegetative cells and autospores of the European *Jenufa* strain SAG 2383 isolated from a soil sample from Swabian Alb (Germany), (b) vegetative cell of *Xylochloris* strain SAG 2382 exhibiting the presence of one pyrenoid, (c) irregular cylindrical to oval autospores typical for the strain, and (d) vegetative cells with lobed margins of the chloroplast. Scale bar represents 20 µm.

Substrate preferences of *Jenufa* and *Xylochloris*

Jenufa (Chlorophyceae) and *Xylochloris* (Trebouxiophyceae) represent distinct lineages of terrestrial green algae without any known members from freshwater habitats. Despite *Jenufa* and *Xylochloris* were detected in both habitats, soils and tree barks, in the tropics, the sequence analysis of their closest relatives from Europe suggests a pattern reflecting different substrate preferences for both genera. In our study *Jenufa* was recovered from hard substrates, except for one isolate (SAG 2383) established from soil. The hard substrates were epilithic biofilms (surface of bunker walls on Helgoland, outer surface of walls of castle Gleichen, walls of Altamira cave in Spain) as well as endolithic biofilms (rocks in Piora Valley/1,965 m a.s.l./Swiss Alps; Horath and Bachofen 2009), or and the upper side of wall scales on castle Gleichen) Although the NMDS analysis (**Fig. 5**) of European partial sequences did not point out any clear groupings due to substrate type (endolithic *versus* epilithic/soil), the second ordination axis indicated variation among sequences due to different light intensities at the sampling sites. This may be seen among *Jenufa* sequences from the Helgoland bunker monument. Two sequences, (KB2B11-06 and SAG 2379, originating from two sites of low irradiance within the bunker were genetically closer than both to another *Jenufa* sequence, KB4B5-06, from a site of three times higher irradiance (HR unpublished results). In addition, the genetic differences between two *Jenufa* sequences, 3GA1K3658 from epilithic and GS1K32 from endolithic habitats of the Gleichen castle sandstone walls, may be explained by adaptation to different light intensities as well, as pointed out by the NMDS-plot (**Fig. 5**). The newly determined *Xylochloris* sequences were all recovered from tree bark as well as from soils. The NMDS-analysis pointed out a separation of two groups of very closely related sequences from tree bark against from those from soils (**Fig. 6**). These two groups may represent even two separate taxa at the level of species or below, as already discussed

above. Members of both groups were recovered also from Ecuador, hence both may be widespread as well.

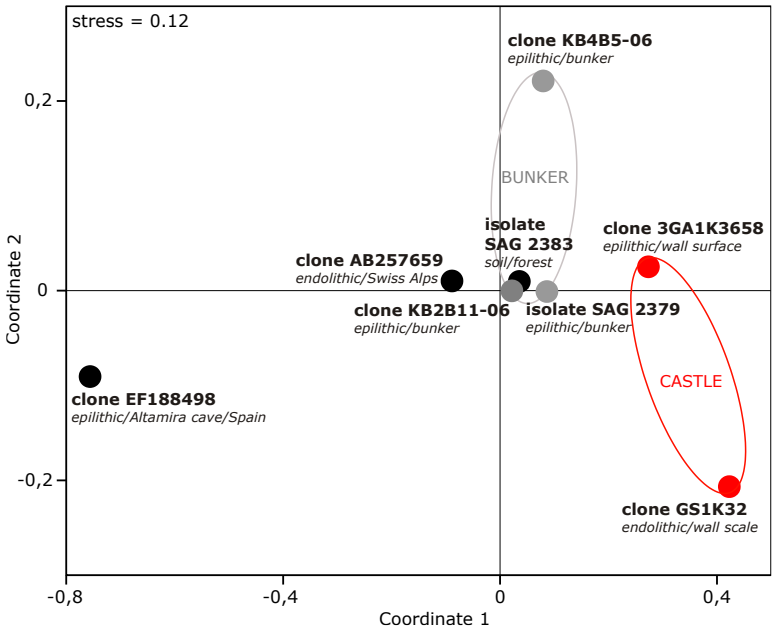

Figure 5. Nonmetric multidimensional Scaling (NMDS) diagram. The ordination analysis based on the Jukes-Cantor genetic distances among six environmental clones and two isolates representing sequences of *Jenufa minuta* detected in Europe on various hard substrates. For the analysis, 18S partial sequences comprising hypervariable regions V2, V3, and V4 were used. The red-colored points represent sequences from the walls of castle Gleichen/Germany, the blue ones are from the bunker on Helgoland, an offshore island in the North Sea. For the corresponding accession numbers of the sequences see **Table 1**.

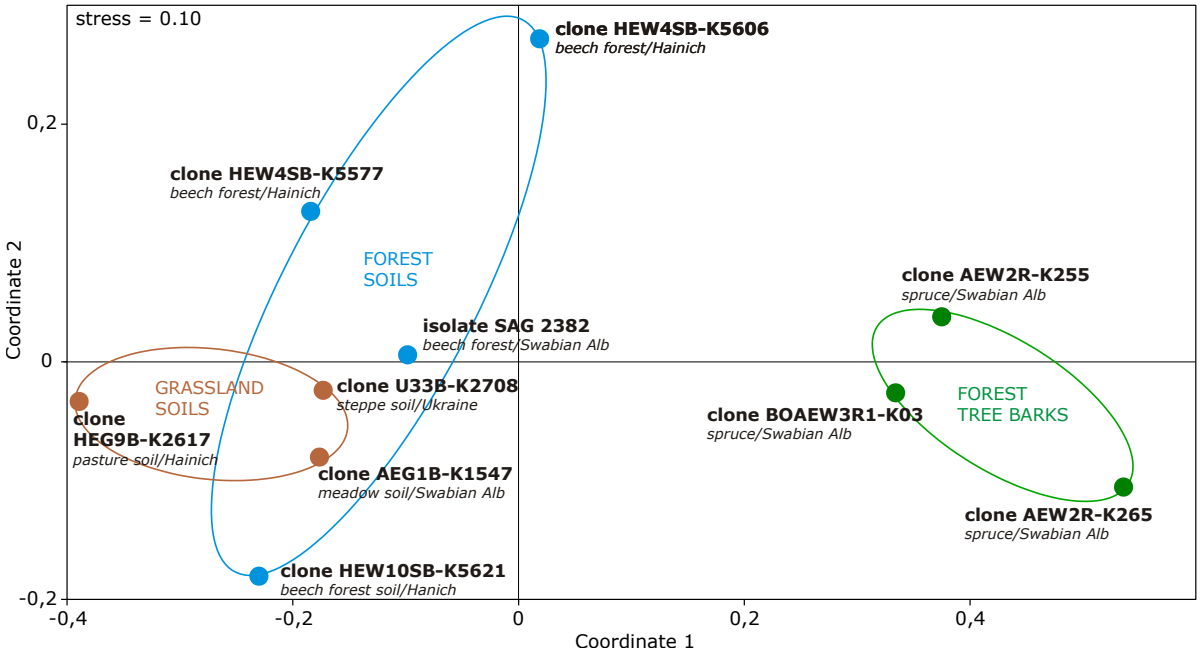

Figure 6. Nonmetric multidimensional scaling (NMDS) diagram. The ordination based on Jukes-Cantor genetic distances among nine environmental clones and one1 isolate representing a yet undescribed European species of the genus *Xylochloris*. For the analysis 18S partial sequences comprising hypervariable regions V2, V3, and V4 were used. The colors encode for three different habitats: green—tree bark, blue—forest soil, brown—grassland soil. For the corresponding accession numbers of the sequences see **Table 1**.

Identification of environmental rDNA clones using new cultured isolates

A high 18S rDNA sequence similarity between strains of closely related terrestrial green algae from tropics and temperate habitats has already been presented recently (Němcová *et al.* 2011). Two cultured isolates of *J. minuta* from the tropics were found closely related to hitherto unidentified environmental clones from the Swiss Alps. This example shows that unidentified environmental clones may become identified as soon as cultures of close relatives become available, at least at the generic level if a morphology-based species or generic concept is applied. In our study we revealed two new isolates, SAG 2379 and SAG 2383, whose sequences were almost identical (p-distances 0.001-0.004) to the environmental clones from the Swiss Alps. Another case where environmental clones became identified due to the availability of culture strains was found in the "*Dictyochloropsis*" clade, sister with *Xylochloris* in the Trebouxiophyceae (**Fig. 3d**). Due to the very low genetic distances (p-distances 0.001 – 0.005) present within the clade, the strains of *Dictyochloropsis* from tree barks in Germany and the environmental clones from the high Andes Mountains (Chile) may be regarded as member of a single species which again exhibits a wide geographic distribution. The genetic distances between the Andes clones/*Dictyochloropsis* pairs were even shorter (0.003-0.004) than between both Andes environmental clones (0.005). In conclusion, for a better understanding of the biogeography of terrestrial microalgae it seems to be of crucial importance not only to sequence environmental clones from somehow exotic or extreme habitats, but also to establish and sequence cultures from seemingly well investigated regions like Central Europe. For the studies on biogeography of terrestrial algae we are still faced with the lack of sufficient molecular data to compare new findings to, because the most diversity evidences from either Central Europe or many other geographical regions relies on morphological observations only (Hoffmann *et al.* 2007; Neustupa and Škaloud 2008; Büdel *et al.* 2009; Khaybullina *et al.* 2010). We expect the situation to change in the future through extended application of the next generation sequencing (e.g. 454-pyrosequencing) concentrated on hypervariable regions of the 18S rDNA (Amaral-Zettler *et al.* 2009).

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General Summary

Green microalgae (Chlorophyta) dominate soils in the northern temperate climate zone, however, their biodiversity is still poorly understood. Available diversity inventories rely on light microscopy and morphospecies, which are hardly distinguishable without molecular markers. Whereas particular attention was paid to molecular diversity of terrestrial microalgae in extreme regions, temperate climate zones remain almost unexplored. We aim to uncover the phylogenetic diversity of green microlagae isolated from Central European soils and from periodically desiccating freshwater creek biofilms. Such terrestrial and semi-aquatic habitats are inhabited by green microalgae, which are presumably able of long-distance dispersal. Their cosmopolitan distribution is presumed, however, supporting molecular evidence is almost missing. By assembling newly obtained green algal sequences together with accessions from remote geographic regions, we further aim to address a question of biogeography of terrestrial microalgae.

Soil samples were taken from grassland and forest plots within the German Biodiversity Exploratories. Freshwater biofilms dominated by green microalgae were sampled in two karstwater creeks in Germany. In total, 280 new monoclonal cultures of green microalgae were examined by molecular phylogenetic methods and by light microscopy. By using ribosomal 18S and ITS2 sequences, we recognized about 100 monophyletic species of green microalgae. The newly obtained sequences were blasted against public databases in order to infer taxonomy, distribution and ecology of the detected species. Further monoclonal cultures and environmental clones originated from additional samplings in Germany, Ecuador, the Arctic and Antarctic.

Most Chlorophyta isolated from German soils were highly similar (≥ 99.5% threshold) to cultured relatives already known from Europe, predominantly from soils and further terrestrial substrates such as tree barks and rocks. Considering a lower similarity threshold (≥ 99%), about 90% of our cultures matched environmental clones inferred from various terrestrial and aquatic habitats. Similarly, Chlorophyta detected in creek biofilms partly represent terrestrial species, but some are known also from planktonic communities. In soils, we detected novel species mostly within established lineages. Only one soil isolate, provisionally named *Navichloris fusiformis*, was recognized as a member of a novel monophyletic lineage, comprising accessions from South and North American deserts. In multiple cases, molecular data supported close relatedness between the European soil isolates and species from tropics and polar regions. We show, that well supported monophyletic clades of *Stichococcus* exhibit either temperate-polar *or* temperate-

tropical distributions. In contrast, some monophyletic clades of *Chlorella*-like microalgae were so far evidenced only from polar regions and hot deserts. The long-distance dispersal was finally confirmed for particular species of *Stichococcus*, *Chlorella* and *Klebsormidum*.

Forest and grassland soils in Central Europe host species of green microalgae otherwise known from a broad spectrum of terrestrial as well as aquatic habitats. New species and even novel lineages can still be uncovered by using standard culturing techniques. Despite the striking similarity between some European and exotic species, unambiguous molecular evidence of the intraspecific long-distance dispersal is still scant. At the present state of knowledge, our data suggest the existence of biogeography of airborne microalgae.

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Appendix | Chapter 1

Supporting Figures

Figure S1. Microphotographs of mixed cultures (Archaeplastida).

Figure S2. Microphotographs of mixed cultures (Archaeplastida, Stramenopiles, Cryptophyta).

Figure S3. Diatoms in mixed cultures.

Figure S4. Cyanobacteria in mixed cultures.

Figure S5. Diversity in mixed cultures as inferred by light-microscopy.

Figure S6. Geographic evidence of 25 most common Green algal morphotypes.

Supporting Tables

Table S1. List of all analyzed isolates.

Table S2a. List of all detected species belonging to Trebouxiophyceae.

Table S2b. List of all detected species belonging to Chlorophyceae, other green algae and Stramenopiles.

Table S3. List of the closest GenBank-relatives of our detected species.

Table S4a. List of all analyzed full and partial 18S rDNA sequences.

Table S4b. Distribution of the green algal species across the sampling sites.

Table S5. General morphological characteristics of the new isolates.

Table S6a. Diversity of morphospecies in soil drill cores.

Table S6b. Diversity of morphospecies in topsoil samples.

Table S7. Frequency of the morphospecies in soil drill cores.

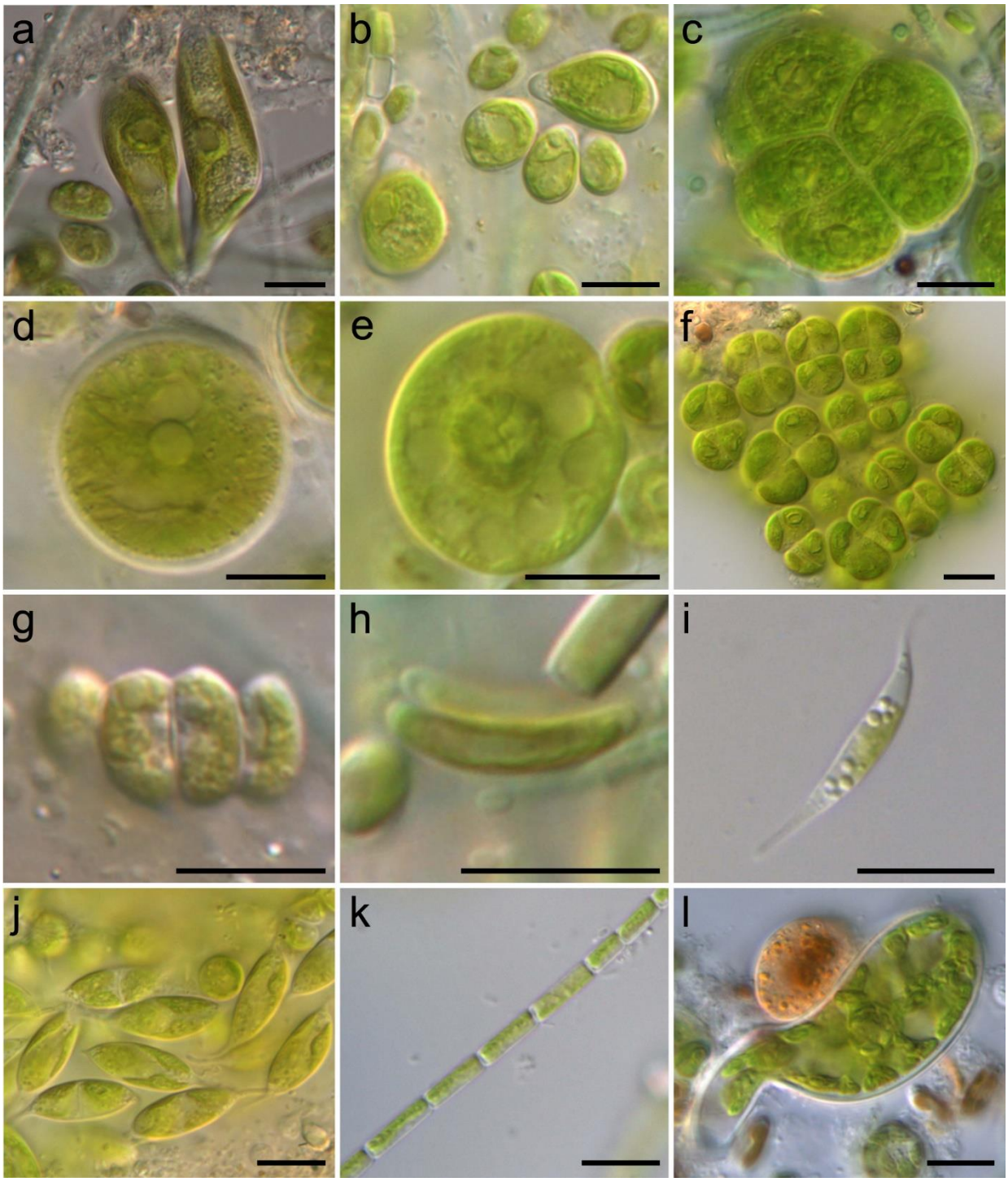


Figure S1. Microphotographs of mixed cultures (Archaeplastida). (a) Characiochloridaceae, *Chlamydopodium* (HEG2); (b) Characiochloridaceae, *Chlamydopodium* (HEG9); (c) cf. *Tetracystis* (HEG9); (d) Actinochloridaceae (HEW1); (e) Actinochloridaceae (HEW1); (f) Chlorosarcinaceae, cf. *Desmotetra* (HEG6); (g) Scenedesmaceae, *Scenedesmus* cf. *soil* (HEG7); (h) Selenastraceae, *Monoraphidium terrestre* (HEG9); (i) cf. *Keratococcus* (HEG7); (j) *Podohedra* (HEG7); (k) cf. *Geminella* (HEW3); (l) *Scotinosphaera* (HEG9). Scale bars = 10 µm.

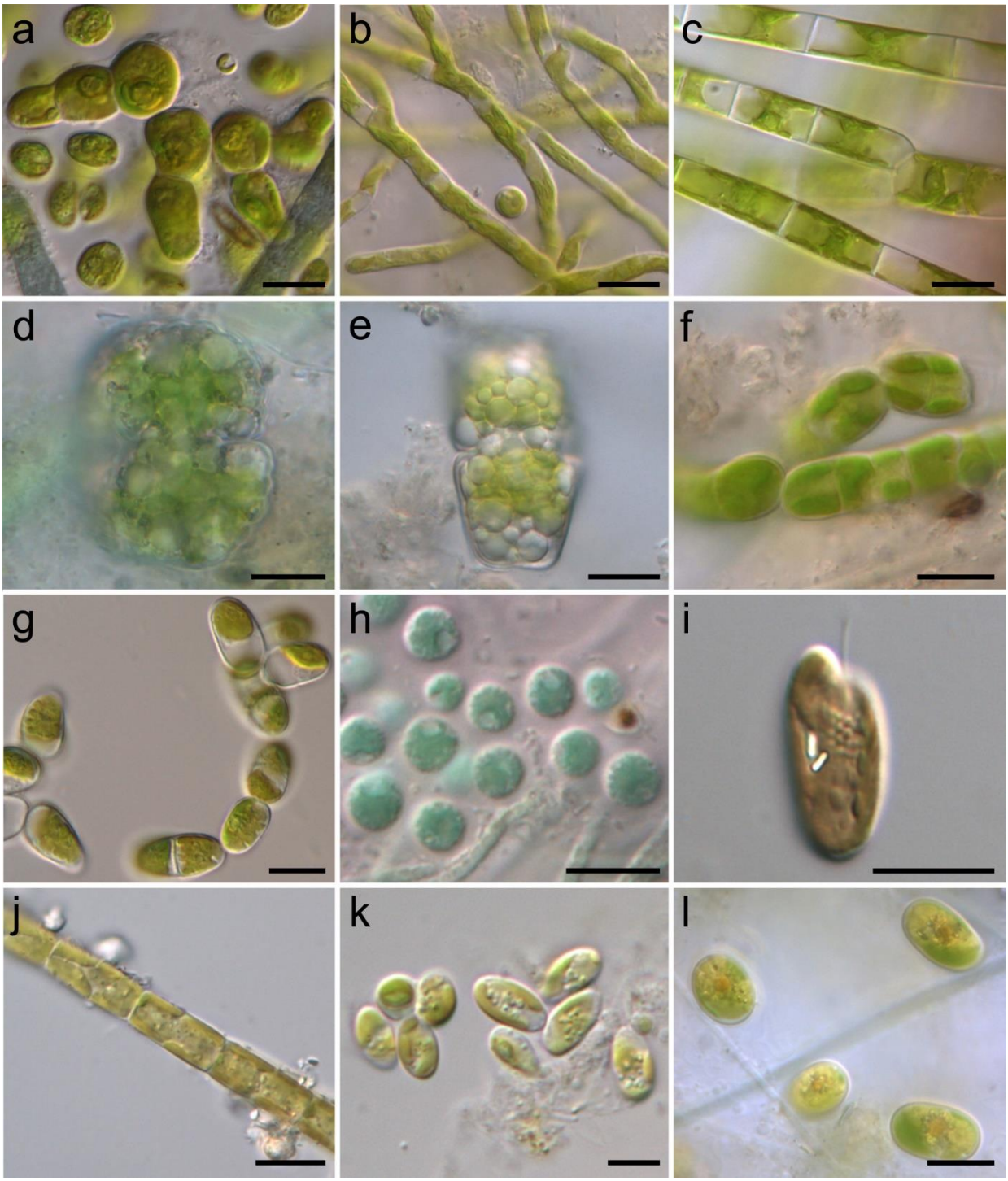


Figure S2. Microphotographs of mixed cultures (Archaeplastida, Stramenopiles, Cryptophyta). (a) cf. *Pseudendoclonium* (HEG7); (b) cf. *Dilabifilum* (SEG8); (c) cf. *Chaetophora* (HEG9); (d) *Cosmarium* (HEG9); (e) *Cosmarium* (HEG9); (f) cf. *Klebsormidium* (HEG9); (g) *Interfilum* cf. *terricola* (HEW); (h) Rhodophyta, *Porphyridium* (HEG9); (i) *Cryptomonas* (HEG9); (j) *Tribonema* (HEG9); (k) cf. *Ellipsoidion* (HEG6); (l) cf. *Monallantus* (HEW4). Scale bars = 10 µm.

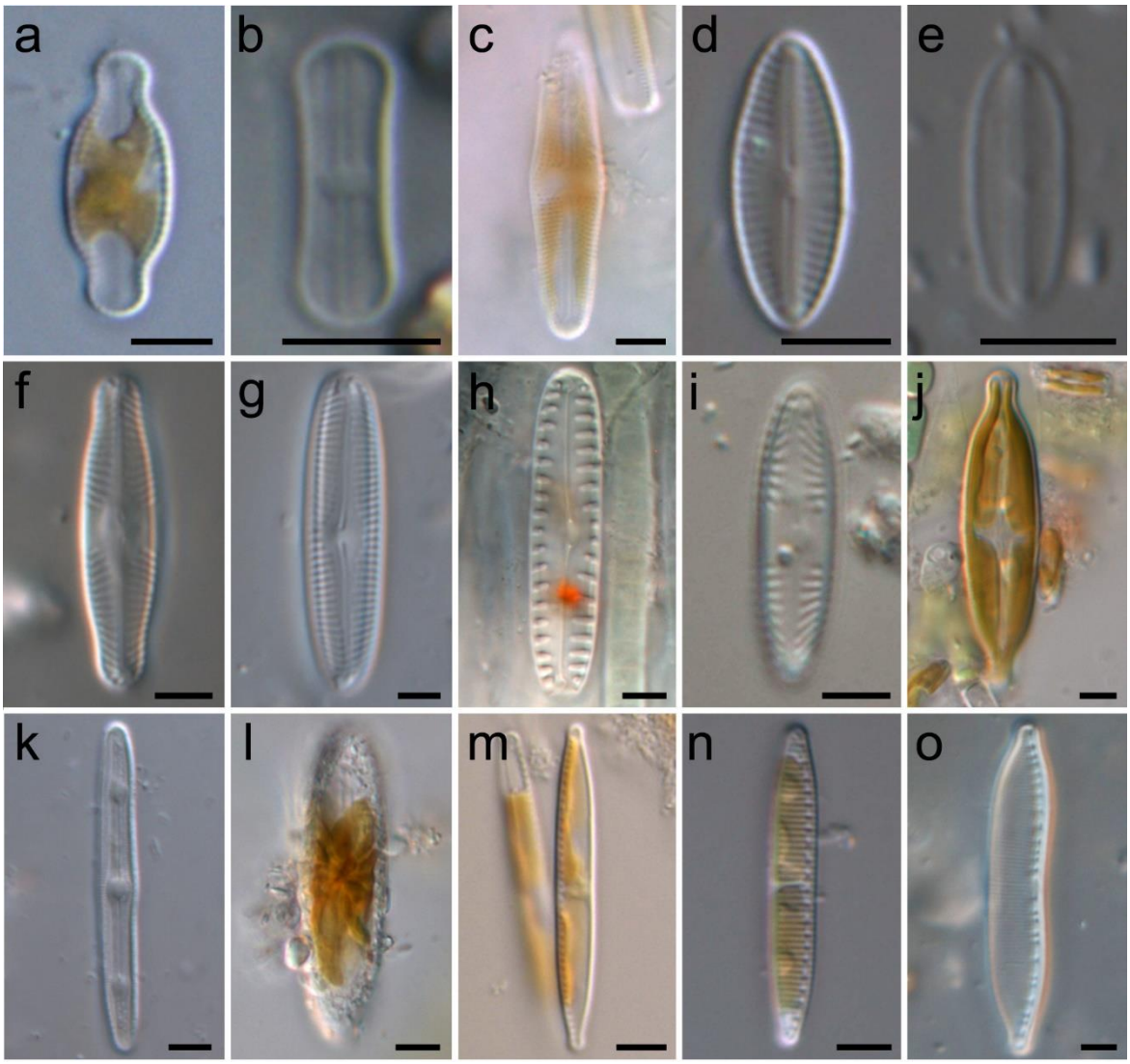


Figure S3. Diatoms in mixed cultures. (a) Monoraphid diatoms, cf. *Planothidium* (HEG9); (b)-(k) Symmetrical biraphid diatoms; (b) *Humidophila* (HEG6); (c) *Luticola* (HEG9); (d) cf. *Eolimna* (HEW3); (e) cf. *Mayamaea* (HEW3); (f) *Pinnularia* (HEW6); (g) *Pinnularia* (HEW9); (h) *Pinnularia* (HEW); (i) *Pinnularia* (HEG8); (j) *Stauroneis* (HEG9); (k) cf. *Neidium* (HEW4); (l) Surirelloid diatoms, *Surirella* (HEW); (m)-(o) Nitzschioid diatoms; (m) *Nitzschia* (HEG4); (n) *Nitzschia* (HEG9); (o) *Hantzschia* (HEG6). Scale bars = 5 µm.

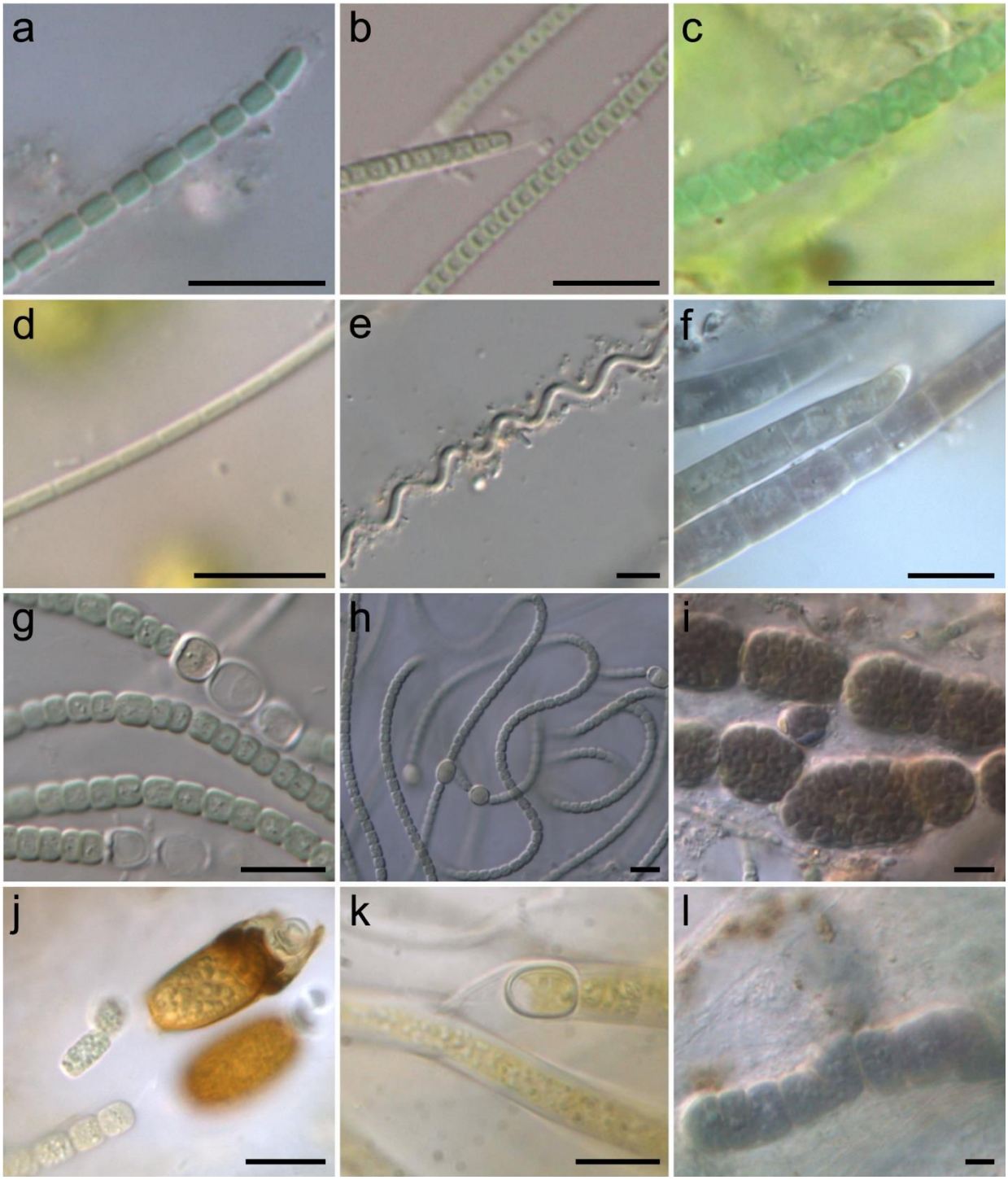


Figure S4. Cyanobacteria in mixed cultures. (a) cf. *Pseudanabaena* (HEG9); (b) cf. *Leptolynbgya* (HEW4); (c) cf. *Leptolyngbya* (HEG9); (d) cf. *Limnothrix* (HEG6); (e) Pseudanabaenales (HEG7); (f) *Phormidium* (HEG7); (g) *Nostoc* (HEG7); (h) *Nostoc* (HEG7); (i) *Nostoc* (HEG7); (j) *Cylindrospermum* (HEG6); (k) cf. *Tolypothrix* (HEG2); (l) cf. *Stigonema* (HEG9). Scale bars = 10 µm.

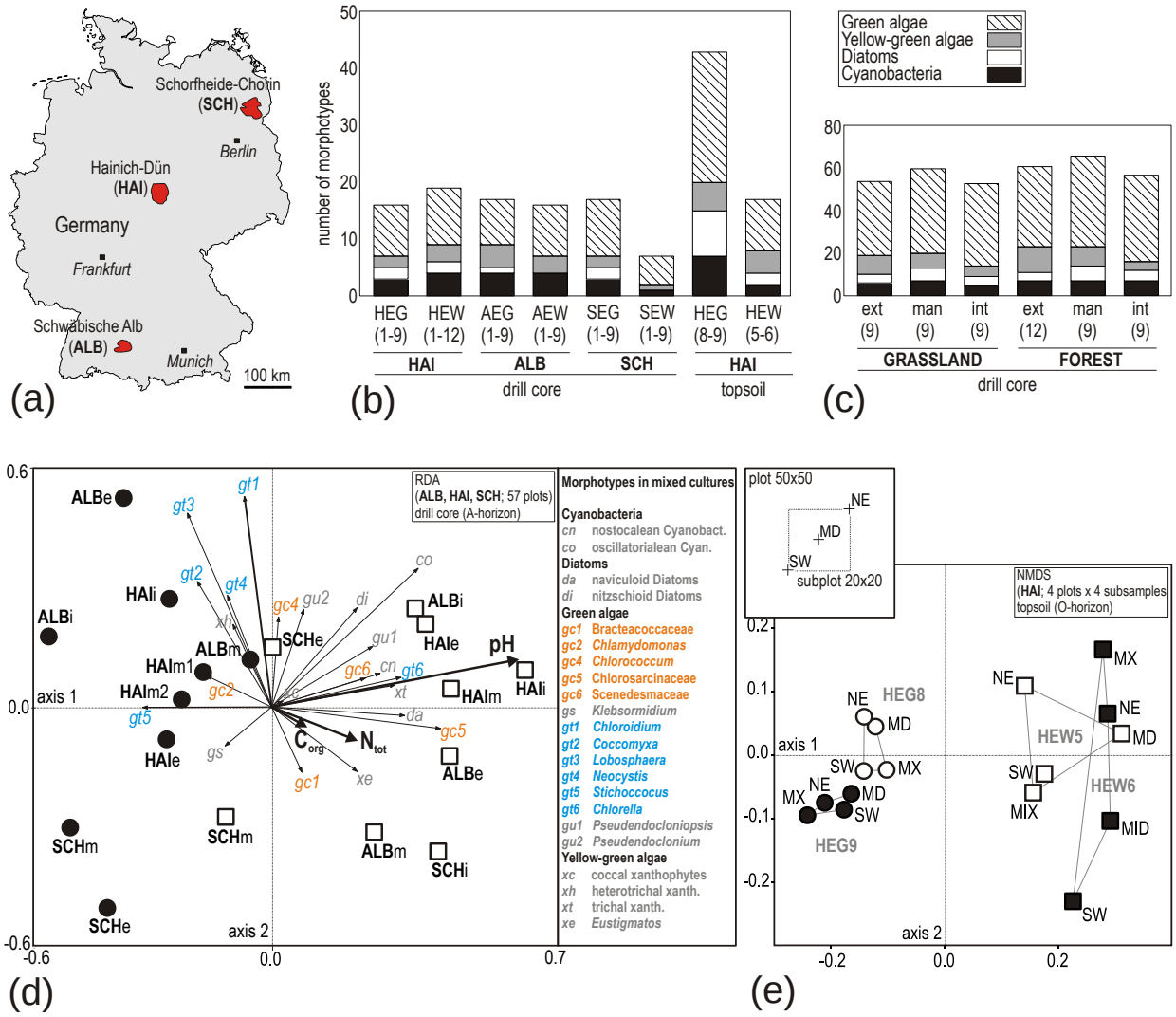


Figure S5. Diversity in mixed cultures as inferred by light-microscopy. (a) Localization of the three German Biodiversity Exploratories. (b) Comparison of alpha-diversities (= numbers of morphotypes) of soil algae in grasslands and forests. Each bar represents pooled data from nine to twelve plots (e.g., HEG(1-9) includes observations from all Hainich grassland plots; (c) Comparison of alpha-diversities among the land-uses (ext=extensive, man=managed, int=intensive). Each bar includes pooled data from multiple plots (the number is given in parentheses). (d) Redundancy analysis (RDA) of algal communities changing along environmental gradients (pH, C_{org}=organic carbon, N_{tot}=total nitrogen). Dots (forests) and squares (grasslands) represent algal communities which were pooled within each land-use category (e.g., ALBe includes observations from plots AEW7-AEW9; e=extensively used plots). A subset of 22 most frequently observed morphotypes are shown (black vectors). (e) Non-metric multidimensional scaling (NMDS) of algal communities in multiple subsamples within a plot. Each dot (forest subsample) and square (grassland subsample) represents data recorded during three seasons. Within each plot (HEG8, HEG9, HEW5, HEW6) three subsamples were analyzed (NE=north-east plot edge, MID=middle point, SW=south-west plot edge, MIX=pooled sample combining all three subsamples within a plot; sampling scheme is shown above).

Figure S6. Geographic evidence of 25 most common Green algal morphotypes determined in German soils and further 46 published species lists (the map shows a selection of 21 studies). ***1*** Germany (this thesis); ***2*** Russia (Patova and Dorokhova 2008); ***3*** Svalbard (Kim et al. 2008); ***4*** Ellesmere Island (Elster et al. 1999); ***5*** Ecuador (Spitzer 2013); ***6*** Samoa (MacEntee et al. 1977); ***6a*** Tahiti (MacEntee et al. 1977); ***6b*** Malaysia (MacEntee et al. 1977); ***6c*** Australia (MacEntee et al. 1977); ***6d*** New Zealand (MacEntee et al. 1977); ***6e*** Fiji (MacEntee et al. 1977); ***7*** Eniwetok Atoll (Arvik and Willson 1974); ***8*** Namibia (Budel et al. 2009); ***9*** China (Chunxiang and Yongding 2003); ***10*** Taiwan (Lin et al. 2013); ***11*** India (Ray and Thomas 2012); ***12*** Saudi Arabia (Al-Fredan and Fathi 2007); ***13*** Siberia (Androsova 1967); ***14*** Antarctica (Cavacini 2001); ***15*** Antarctica (Fermani et al. 2007); ***16*** Washington (Fairchild and Willson 1967); ***17*** Great Smoky Mts. (Khaybullina et al. 2010); ***18*** New Jersey (Hunt et al. 2008); ***20*** Mexico (Flechtner et al. 1998b); ***21*** Porto Rico (Archibald 1972). The percentages given for morphotypes correspond to their occurrence within the set of 46 species lists mentioned on pages 2-3.

Table S1. List of all analyzed isolates.

Species	Identifier	GPS	Exploratory	Plot	Habitat	Sampling	18S	ITS2
Acutodesmus rubescens	LH08SG8041	N53° 6' 50.294" E14° 1' 1.559"	SCH	SEG8	grassland	drill core	1656	-
Bracteacoccus cohaerens	LH10HG9034	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1722	-
Bracteacoccus cohaerens (cf.)	LH08SG2015	N53° 5' 21.505" E13° 58' 48.169"	SCH	SEG2	grassland	drill core	2056	-
Chlamydomonas gerloffii (cf.)	LH08SW5031	N53° 3' 25.321" E13° 53' 7.318"	SCH	SEW5	forest	drill core	1711	-
Chlamydomonas rapa	LH08SG1077	N53° 5' 14.712" E13° 58' 10.717"	SCH	SEG1	grassland	drill core	1694	-
Chlamydomonas rapa	LH08SG9055	N53° 5' 53.455" E13° 36' 45.241"	SCH	SEG9	grassland	drill core	1660	-
Chlamydomonas rapa (cf.)	LH10HG1027	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	soil surface	1710	290
Chlamydomonas typica (cf.)	LH08SG9022	N53° 5' 53.455" E13° 36' 45.241"	SCH	SEG9	grassland	drill core	1262	-
Chlamydopodium vacuolatum	LH10HG1013	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	soil surface	1728	287
Chlorococcum minutum (cf.)	LH08AG701K	N48° 23' 29.116" E9° 22' 36.65"	ALB	AEG7	grassland	drill core	793	-
Chlorococcum minutum (cf.)	LH08AW5056 (=SAG 2479)	N48° 25' 10.626" E9° 24' 52.854"	ALB	AEW5	forest	drill core	1707	-
Chlorococcum minutum (cf.)	LH08AW5107	N48° 25' 10.626" E9° 24' 52.854"	ALB	AEW5	forest	drill core	1662	-
Chlorococcum minutum (cf.)	LH08AW5111	N48° 25' 10.626" E9° 24' 52.854"	ALB	AEW5	forest	drill core	1346	-
Chlorococcum sphacosum	LH10HG3113	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	soil surface	1713	-
Coelastrella multistriata	LH08AG2003	N48° 22' 36.686" E9° 28' 22.023"	ALB	AEG2	grassland	drill core	535	-
Coelastrella multistriata	LH08AW4118	N48° 23' 56.755" E9° 14' 41.378"	ALB	AEW4	forest	drill core	1208	-
Coelastrella multistriata	LH10HG7083	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	2117	-
Coelastrella multistriata	LH10HG7097	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	512	-
Coelastrella multistriata	LH10HG7100	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	700	-
Coelastrella multistriata	LH10HG7102	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	411	-
Coelastrella multistriata	LH10HG8109	N51° 16' 16.527" E10° 25' 4.6"	HAI	HEG8	grassland	soil surface	454	-
Coelastrella sp.	LH10HG1009	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	soil surface	1770	-
Coelastrella sp.	LH10HG1033	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	soil surface	1622	-
Coelastrella sp.	LH10HG2087	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	470	-
Coelastrella sp.	LH10HG2098	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	2206	-
Coelastrella sp.	LH10HG2P01	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	589	-
Coelastrella sp.	LH10HG2P12	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	599	-
Coelastrella sp.	LH10HG5136	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	soil surface	1313	-
Coelastrella sp.	LH10HG6035	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1511	-
Coelastrella sp.	LH10HG6060	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1616	266
Coelastrella sp.	LH10HG7017	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1683	-
Coelastrella sp.	LH10HG7018	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1429	297
Coelastrella sp.	LH10HG7023	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1637	-
Coelastrella sp.	LH10HG7030	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1506	298
Coelastrella sp.	LH10HG9130	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1053	-
Desmotetra stigmatica	LH08SG2049	N53° 5' 21.505" E13° 58' 48.169"	SCH	SEG2	grassland	drill core	1667	-

Table S1. (continuation)

Species	Identifier	GPS	Exploratory	Plot	Habitat	Sampling	18S	ITS2
Desmotetra stigmatica	LH10HG6P18	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	494	-
Heterochlamydomonas sp.	LH08AG2004	N48° 22' 36.686" E9° 28' 22.023"	ALB	AEG2	grassland	drill core	1699	-
Jenufa sp.	LH08AW8035 (=SAG 2383)	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1700	-
Jenufa sp.	LH08AW8098	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1277	-
Oogamochlamys sp.(I)	LH08SG8047	N53° 6' 50.294" E14° 1' 1.559"	SCH	SEG8	grassland	drill core	1654	-
Oogamochlamys sp.(II)	LH08AW1069 (=SAG 2476)	N48° 28' 41.063" E9° 20' 3.877"	ALB	AEW1	forest	drill core	1279	-
Pseudomuriella aurantiaca	LH10HG2039	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1656	-
Pseudomuriella aurantiaca	LH10HG9038	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	2081	-
Stephanosphaerinia sp.	LH10HG6108	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1719	-
Tatrensinia sp.(I)	LH08SW7115	N53° 6' 26.453" E13° 41' 39.908"	SCH	SEW7	forest	drill core	1716	-
Tatrensinia sp.(II)	LH10HG7016	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1733	-
Tatrensinia sp.(II)	LH10HG9131	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	527	-
Pedinomonas minor (cf.)	LH08SG2033	N53° 5' 21.505" E13° 58' 48.169"	SCH	SEG2	grassland	drill core	1489	-
Auxenochlorella protothecoides	LH10HG5119	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	soil surface	1711	-
Auxenochlorella protothecoides	LH10HG6096	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1725	-
Auxenochlorella protothecoides	LH10HG7124	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1720	-
Auxenochlorella sp.	LH08AW4103 (=SAG 2478)	N48° 23' 56.755" E9° 14' 41.378"	ALB	AEW4	forest	drill core	1655	321
Chlorella mirabilis (cf.)	LH08AG9040	N48° 23' 40.815" E9° 30' 10.053"	ALB	AEG9	grassland	drill core	1648	314
Chlorella mirabilis (cf.)	LH10HG6139	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1683	-
Chlorella vulgaris	LH08HG1081	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	drill core	1687	300
Chlorella vulgaris	LH08HG2013	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	drill core	1692	-
Chlorella vulgaris	LH08HG2065	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	drill core	1695	-
Chlorella vulgaris	LH08HG2083	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	drill core	1676	-
Chlorella vulgaris	LH08HG2091	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	drill core	1695	-
Chlorella vulgaris	LH08HG2096	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	drill core	1678	-
Chlorella vulgaris	LH08HG4032	N51° 6' 48.104" E10° 26' 10.249"	HAI	HEG4	grassland	drill core	1671	300
Chlorella vulgaris	LH08HG4088	N51° 6' 48.104" E10° 26' 10.249"	HAI	HEG4	grassland	drill core	1692	300
Chlorella vulgaris	LH08HG5074	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	drill core	1695	300
Chlorella vulgaris	LH08HG5082	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	drill core	1692	300
Chlorella vulgaris	LH08HW6087	N51° 16' 3.791" E10° 14' 21.762"	HAI	HEW6	forest	drill core	303	-
Chlorella vulgaris	LH08HW9060	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	drill core	1686	-
Chlorella vulgaris	LH08HW9094	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	drill core	1686	300
Chlorella vulgaris	LH08SG1071	N53° 5' 14.712" E13° 58' 10.717"	SCH	SEG1	grassland	drill core	1695	300
Chlorella vulgaris	LH08SG3006	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	1695	300
Chlorella vulgaris	LH10HG2049	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1720	300
Chlorella vulgaris	LH10HG3088	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	soil surface	1674	-

Table S1. (continuation)

Species	Identifier	GPS	Exploratory	Plot	Habitat	Sampling	18S	ITS2
Chlorella vulgaris	LH10HG4093	N51° 6' 48.104" E10° 26' 10.249"	HAI	HEG4	grassland	soil surface	1724	-
Chlorella vulgaris	LH10HG6014	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1719	300
Chlorella vulgaris	LH10HG6052	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1735	300
Chlorella vulgaris	LH10HG6099	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1665	-
Chlorella vulgaris	LH10HG9075	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1720	300
Chlorella vulgaris (cf.)	LH10HG2081	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1690	305
Chlorella vulgaris (cf.)	LH10HG7072	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1448	-
Chloroidium ellipsoideum (cf.)	LH08AG8046	N48° 25' 21.505" E9° 29' 31.649"	ALB	AEG8	grassland	drill core	1658	-
Chloroidium ellipsoideum (cf.)	LH08AG9062	N48° 23' 40.815" E9° 30' 10.053"	ALB	AEG9	grassland	drill core	1706	-
Chloroidium ellipsoideum (cf.)	LH10HG6086	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1677	-
Chloroidium ellipsoideum (cf.)	LH10HG7132	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1632	-
Chloroidium ellipsoideum (cf.)	LH10HG9105	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1724	-
Chloroidium saccharophilum	LH08AW8042	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1694	-
Chloroidium saccharophilum	LH10HG5089	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	soil surface	568	-
Chloroidium saccharophilum	LH10HG7062	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1719	-
Coccomyxa viridis	LH08AW8039 (=SAG 2483)	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1731	-
Coccomyxa simplex	LH08SG9051	N53° 5' 53.455" E13° 36' 45.241"	SCH	SEG9	grassland	drill core	1409	-
Coccomyxa sp.	LH08AW1017	N48° 28' 41.063" E9° 20' 3.877"	ALB	AEW1	forest	drill core	1732	-
Dictyochloropsis splendida	LH08AW3050	N48° 24' 44.145" E9° 21' 20.127"	ALB	AEW3	forest	drill core	1722	-
Diplosphaera sp.(I)	LH08HW8075	N51° 21' 20.852" E10° 31' 1.083"	HAI	HEW8	forest	drill core	1824	326
Diplosphaera sp.(II)	LH08AG9089	N48° 23' 40.815" E9° 30' 10.053"	ALB	AEG9	grassland	drill core	1660	334
Lobosphaera bisecta (cf.)	LH10HG3P15	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	soil surface	601	-
Lobosphaera irregularis (cf.)	LH08AW3064	N48° 24' 44.145" E9° 21' 20.127"	ALB	AEW3	forest	drill core	1731	-
Lobosphaera irregularis (cf.)	LH08SW5063	N53° 3' 25.321" E13° 53' 7.318"	SCH	SEW5	forest	drill core	705	-
Muriella terrestris (cf.)	LH08SG3009	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	1692	312
Muriella terrestris (cf.)	LH10HG1070	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	soil surface	1767	-
Muriella terrestris (cf.)	LH10HG1076	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	soil surface	1732	312
Muriella terrestris (cf.)	LH10HG7118	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1675	-
Muriella terrestris (cf.)	LH10HG8050	N51° 16' 16.527" E10° 25' 4.6"	HAI	HEG8	grassland	soil surface	1733	311
Muriella terrestris (cf.)	LH10HG9077	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1729	-
Nannochloris sp.	LH08SG3102	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	802	-
Nannochloris sp.	LH08SG8030	N53° 6' 50.294" E14° 1' 1.559"	SCH	SEG8	grassland	drill core	1648	-
Nannochloris sp.	LH10HG6095	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1721	-
Navichloris fusiformis (provisional denomination)	LH08AW3007 (=SAG 2477)	N48° 24' 44.145" E9° 21' 20.127"	ALB	AEW3	forest	drill core	1694	-
Neocystis brevis	LH08AG9012	N48° 23' 40.815" E9° 30' 10.053"	ALB	AEG9	grassland	drill core	1673	-
Neocystis brevis	LH08AW8001 (=SAG 2480)	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1735	-

Table S1. (continuation)

Species	Identifier	GPS	Exploratory	Plot	Habitat	Sampling	18S	ITS2
Neocystis brevis	LH08HW6059	N51° 16' 3.791" E10° 14' 21.762"	HAI	HEW6	forest	drill core	1713	-
Neocystis brevis	LH08HW6108	N51° 16' 3.791" E10° 14' 21.762"	HAI	HEW6	forest	drill core	1687	-
Neocystis brevis	LH08SG2072	N53° 5' 21.505" E13° 58' 48.169"	SCH	SEG2	grassland	drill core	1696	-
Neocystis brevis	LH10HG4082	N51° 6' 48.104" E10° 26' 10.249"	HAI	HEG4	grassland	soil surface	357	-
Neocystis brevis	LH10HG9054	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1731	-
Neocystis brevis	LH10HG9080	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1736	295
Neocystis brevis	LH10HG9P02	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	543	-
Pseudostichococcus monallantoides	LH08AG7097	N48° 23' 29.116" E9° 22' 36.65"	ALB	AEG7	grassland	drill core	392	-
Pseudostichococcus monallantoides	LH10HG2066	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1709	-
Pseudostichococcus monallantoides	LH10HG3045	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	soil surface	1737	277
Pseudostichococcus sp.	LH08SW8044	N53° 11' 30.47" E13° 55' 49.216"	SCH	SEW8	forest	drill core	1661	285
Stichococcus sp.(I)	LH08AG9028	N48° 23' 40.815" E9° 30' 10.053"	ALB	AEG9	grassland	drill core	1245	-
Stichococcus sp.(I)	LH08AW8025	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1712	-
Stichococcus sp.(II)	LH08AG1100	N48° 23' 52.818" E9° 20' 31.152"	ALB	AEG1	grassland	drill core	573	-
Stichococcus sp.(II)	LH08SG5057	N53° 6' 26.83" E14° 0' 1.885"	SCH	SEG5	grassland	drill core	1695	302
Stichococcus sp.(II)	LH08SG5067	N53° 6' 26.83" E14° 0' 1.885"	SCH	SEG5	grassland	drill core	1659	-
Stichococcus sp.(II)	LH08SG5079	N53° 6' 26.83" E14° 0' 1.885"	SCH	SEG5	grassland	drill core	1692	302
Stichococcus sp.(II)	LH08SG5090	N53° 6' 26.83" E14° 0' 1.885"	SCH	SEG5	grassland	drill core	1683	302
Stichococcus sp.(II)	LH08SG5092	N53° 6' 26.83" E14° 0' 1.885"	SCH	SEG5	grassland	drill core	785	-
Stichococcus sp.(II)	LH10HG2063	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1721	302
Stichococcus sp.(II)	LH10HG2067	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1721	-
Stichococcus sp.(II)	LH10HG3128	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	soil surface	1721	-
Stichococcus sp.(II)	LH10HG7090	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1719	302
Stichococcus sp.(II)	LH10HG7092	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1710	-
Stichococcus sp.(II)	LH10HG9068	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1722	-
Stichococcus sp.(III)	LH08AG7010	N48° 23' 29.116" E9° 22' 36.65"	ALB	AEG7	grassland	drill core	1660	274
Stichococcus sp.(III)	LH10HG6110	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	soil surface	1724	-
Stichococcus sp.(IV)	LH08SG1073	N53° 5' 14.712" E13° 58' 10.717"	SCH	SEG1	grassland	drill core	1696	325
Stichococcus sp.(V)	LH08SW1099	N52° 54' 3.05" E13° 50' 46.921"	SCH	SEW1	forest	drill core	1653	310
Stichococcus sp.(VI)	LH08AW8023 (=SAG 2482)	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1722	311
Stichococcus sp.(VI)	LH08AW8104	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1687	311
Stichococcus sp.(VII)	LH08AW8002 (=SAG 2481)	N48° 22' 57.322" E9° 22' 56.584"	ALB	AEW8	forest	drill core	1730	-
Unidentified Chlorellaceae (I)	LH08AG1034	N48° 23' 52.818" E9° 20' 31.152"	ALB	AEG1	grassland	drill core	1681	274
Unidentified Chlorellaceae (I)	LH10HG7073	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1409	-
Unidentified Chlorellaceae (II)	LH08SG2053	N53° 5' 21.505" E13° 58' 48.169"	SCH	SEG2	grassland	drill core	1672	-
Unidentified Chlorellaceae (II)	LH08SG3029	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	798	-

Table S1. (continuation)

Species	Identifier	GPS	Exploratory	Plot	Habitat	Sampling	18S	ITS2
Unidentified Chlorellaceae (II)	LH10HG2094	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1719	-
Unidentified Chlorellaceae (II)	LH10HG3135	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	soil surface	1718	-
Unidentified Chlorellaceae (II)	LH10HG7071	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1732	-
Unidentified Chlorellaceae (II)	LH10HG9020	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1617	299
Unidentified Chlorellaceae (II)	LH10HG9026	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	soil surface	1716	274
Unidentified Chlorellaceae (III)	LH08SG3078	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	1611	299
Unidentified Chlorellaceae (III)	LH08SG3093	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	1678	299
Unidentified Chlorellaceae (IV)	LH10HG709K	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1685	-
Xylochloris sp.	LH08AG7024 (=SAG 2382)	N48° 23' 29.116" E9° 22' 36.65"	ALB	AEG7	grassland	drill core	1719	-
Pseudendocloniopsis botryoides	LH08HW9058	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	drill core	2058	-
Klebsormidium dissectum/elegans (cf.)	LH08AG1113	N48° 23' 52.818" E9° 20' 31.152"	ALB	AEG1	grassland	drill core	2038	-
Klebsormidium dissectum/elegans (cf.)	LH08AG7011	N48° 23' 29.116" E9° 22' 36.65"	ALB	AEG7	grassland	drill core	2236	-
Klebsormidium dissectum/elegans (cf.)	LH08AG9045	N48° 23' 40.815" E9° 30' 10.053"	ALB	AEG9	grassland	drill core	2093	-
Klebsormidium dissectum/elegans (cf.)	LH08HW1038	N51° 11' 7.278" E10° 19' 25.036"	HAI	HEW1	forest	drill core	1882	274
Klebsormidium dissectum/elegans (cf.)	LH08HW4109	N51° 22' 10.134" E10° 31' 59.979"	HAI	HEW4	forest	drill core	2234	-
Klebsormidium dissectum/elegans (cf.)	LH08HW5021	N51° 15' 49.961" E10° 14' 27.448"	HAI	HEW5	forest	drill core	1827	-
Klebsormidium dissectum/elegans (cf.)	LH08HW9005	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	drill core	1736	-
Klebsormidium dissectum/elegans (cf.)	LH08HW9106	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	drill core	2211	274
Klebsormidium flaccidum (cf.)	LH08HW5061	N51° 15' 49.961" E10° 14' 27.448"	HAI	HEW5	forest	drill core	2064	274
Klebsormidium flaccidum (cf.)	LH08SG3027	N53° 6' 10.204" E13° 59' 8.519"	SCH	SEG3	grassland	drill core	1817	-
Klebsormidium flaccidum (cf.)	LH10HG2056	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	soil surface	1777	274
Klebsormidium flaccidum (cf.)	LH10HG7028	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	soil surface	1794	275
Asterosiphon sp.	LH10HG3064	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	drill core	1780	-
Botrydiopsalean sp.	LH08AW1076	N48° 28' 41.063" E9° 20' 3.877"	ALB	AEW1	forest	soil surface	1763	-
Botrydiopsalean sp.	LH08SG2K53	N53° 5' 21.505" E13° 58' 48.169"	SCH	SEG2	grassland	soil surface	619	-
Botrydiopsis callosa (cf.)	LH08AW4043	N48° 23' 56.755" E9° 14' 41.378"	ALB	AEW4	forest	soil surface	1753	-
Heterococcus sp.	LH10HG2140	N51° 0' 2.696" E10° 25' 48.036"	HAI	HEG2	grassland	drill core	1734	-
Heterococcus sp.	LH10HG9085	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	1734	-
Heterococcus chodatii (cf.)	LH10HG9111	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	1693	-
Heterococcus chodatii (cf.)	LH10HG9126	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	627	-
Heterococcus caespitosus (cf.)	LH08AG2020	N48° 22' 36.686" E9° 28' 22.023"	ALB	AEG2	grassland	soil surface	1690	-
Heterothrix sessile	LH10HG5079	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	drill core	1791	-
Heterothrix sessile	LH10HG9037	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	1568	-
Heterothrix sp.	LH08SG5052	N53° 6' 26.83" E14° 0' 1.885"	SCH	SEG5	grassland	soil surface	1779	-
Heterothrix sp.	LH10HG7061	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	drill core	1806	-

Table S1. (continuation)

Species	Identifier	GPS	Exploratory	Plot	Habitat	Sampling	18S	ITS2
Xanthonema bristolianum (cf.)	LH08HW9018	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	soil surface	1752	-
Xanthonema bristolianum (cf.)	LH10HG6059	N51° 12' 53.766" E10° 23' 28.395"	HAI	HEG6	grassland	drill core	1780	-
Xanthonema exile (cf.)	LH10HG7078	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	drill core	1783	-
Xanthonema sp.	LH10HG1K69	N50° 58' 17.934" E10° 24' 19.306"	HAI	HEG1	grassland	drill core	1716	-
Xanthonema sp.	LH10HG3065	N50° 59' 53.129" E10° 25' 58.616"	HAI	HEG3	grassland	drill core	1783	-
Xanthonema sp.	LH10HG7029	N51° 16' 24.897" E10° 24' 37.485"	HAI	HEG7	grassland	drill core	1782	-
Xanthonema sp.	LH10HG8112	N51° 16' 16.527" E10° 25' 4.6"	HAI	HEG8	grassland	drill core	374	-
Xanthonema sp.	LH10HG9031	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	1782	-
Xanthonema sp.	LH10HG9058	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	1782	-
Xanthonema sp.	LH10HW9129	N51° 7' 48.871" E10° 22' 52.139"	HAI	HEW9	forest	drill core	1575	-
Eustigmatos sp.	LH10HG5036	N51° 12' 57.22" E10° 19' 21.096"	HAI	HEG5	grassland	drill core	1764	-
Eustigmatos sp.	LH10HG9133	N51° 13' 26.031" E10° 22' 50.834"	HAI	HEG9	grassland	drill core	1691	-

Legend. ALB=Schwäbische Alb, HAI=Hainich-Dün, SCH=Schorfheide-Chorin.

Table S2a. List of all detected species belonging to Trebouxiophyceae.

Species	Clade	Representative isolates and SAG-strains	GenBank
Auxenochlorella protothecoides	Chlorellaceae	LH10HG6096	-
Auxenochlorella sp.	Chlorellaceae	LH08AW4103 (=SAG 2478)	KP081390
Chlorella mirabilis (cf.)	*Prasiola*	LH08AG9040	-
Chlorella mirabilis (cf.)	*Prasiola*	LH10HG6139	-
Chlorella vulgaris	Chlorellaceae	LH08HG1081	-
Chlorella vulgaris	Chlorellaceae	LH08SG3006	-
Chlorella vulgaris (cf.)	Chlorellaceae	LH10HG2081 (partial 18S)	-
Chlorella vulgaris (cf.)	Chlorellaceae	LH10HG7072 (partial 18S)	-
Chloroidium ellipsoideum (cf.)	*Watanabea*	LH10HG9105	-
Chloroidium saccharophilum	*Watanabea*	LH10HG7062	-
Coccomyxa viridis	*Botryococcus*	LH08AW8039 (=SAG 2483)	KP081391
Coccomyxa simplex	*Botryococcus*	LH08SG9051 (partial 18S)	-
Coccomyxa sp.	*Botryococcus*	LH08AW1017	KP081392
Dictyochloropsis splendida	incertae sedis	LH08AW3050 (= LH-aw3050)	JQ988930
Diplosphaera sp.(I)	*Prasiola*	LH08HW8075	-
Diplosphaera sp.(II)	*Prasiola*	LH08AG9089	-
Lobosphaera bisecta (cf.)	*Lobosphaera*	LH10HG3P15 (partial 18S)	-
Lobosphaera irregularis (cf.)	*Lobosphaera*	LH08AW3064	KP081398
Muriella terrestris (cf.)	Chlorellaceae	LH08SG3009	-
Nannochloris sp.	Chlorellaceae	LH08SG8030	-
Navichloris fusiformis (proposed taxon)	incertae sedis	LH08AW3007 (=SAG 2477)	KP081399
Neocystis brevis	*Neocystis*	LH10HG9080	-
Pseudostichococcus monallantoides	*Prasiola*	LH10HG3045	-
Pseudostichococcus sp.	*Prasiola*	LH08SW8044	-
Stichococcus sp.(I)	*Prasiola*	LH08AW8025	KP081397
Stichococcus sp.(II)	*Prasiola*	LH08SG5057	-
Stichococcus sp.(III)	*Prasiola*	LH10HG6110, LH08AG7010	-
Stichococcus sp.(IV)	*Prasiola*	LH08SG1073	-
Stichococcus sp.(V)	*Prasiola*	LH08SW1099	-
Stichococcus sp.(VI)	*Prasiola*	LH08AW8023 (=SAG 2482)	KP081395
Stichococcus sp.(VII)	*Prasiola*	LH08AW8002 (=SAG 2481)	KP081394
Unidentified Chlorellaceae (I)	Chlorellaceae	LH08AG1034	-
Unidentified Chlorellaceae (II)	Chlorellaceae	LH10HG9020	-
Unidentified Chlorellaceae (III)	Chlorellaceae	LH08SG3093	-
Unidentified Chlorellaceae (IV)	Chlorellaceae	LH10HG709K	-
Xylochloris sp.	incertae sedis	LH08AG7024 (= SAG 2382)	JQ988942

Table S2b. List of all detected species belonging to Chlorophyceae, other green algae and Stramenopiles.

Species	Clade	Representative isolates and SAG-strains	GenBank
Acutodesmus rubescens	Sphaeropleales	LH08SG8041	-
Bracteacoccus cohaerens	Sphaeropleales	LH10HG9034	-
Bracteacoccus cohaerens (cf.)	Sphaeropleales	LH08SG2015	-
Chlamydomonas gerloffii (cf.)	Chlamydomonadales	LH08SW5031	-
Chlamydomonas rapa	Chlamydomonadales	LH08SG1077	-
Chlamydomonas rapa (cf.)	Chlamydomonadales	LH10HG1027	-
Chlamydomonas typica (cf.)	Chlamydomonadales	LH08SG9022 (partial 18S)	-
Chlamydopodium vacuolatum	Chlamydomonadales	LH10HG1013	-
Chlorococcum minutum (cf.)	Chlamydomonadales	LH08AW5056 (=SAG 2479)	KP081402
Chlorococcum sphacosum	Chlamydomonadales	LH10HG3113	-
Coelastrella multistriata	Sphaeropleales	LH10HG7083	-
Coelastrella sp.	Sphaeropleales	LH10HG2098 (+LH10HG7018)	-
Desmotetra stigmatica	Chlamydomonadales	LH08SG2049	-
Heterochlamydomonas sp.	Chlamydomonadales	LH08AG2004	-
Jenufa sp.	incertae sedis	LH08AW8035 (= SAG 2383)	JQ988933
Oogamochlamys sp.(I)	Chlamydomonadales	LH08SG8047	-
Oogamochlamys sp.(II)	Chlamydomonadales	LH08AW1069 (= SAG 2476; partial 18S)	KP081401
Pseudomuriella aurantiaca	Sphaeropleales	LH10HG2039	-
Stephanosphaerinia sp.	Chlamydomonadales	LH10HG6108	-
Tatrensinia sp.(I)	Chlamydomonadales	LH08SW7115	-
Tatrensinia sp.(II)	Chlamydomonadales	LH10HG7016 (+LH10HG9131)	-
Pseudendocloniopsis botryoides (cf.)	Ulotrichales	LH08HW9058	-
Pedinomonas minor (cf.)	Pedinophyceae	LH08SG2033 (partial 18S)	-
Klebsormidium dissectum/elegans (cf.)	Streptophyta	LH08HW9106	-
Klebsormidium flaccidum (cf.)	Streptophyta	LH10HG2056	-
Asterosiphon sp.	incertae sedis	LH10HG3064	-
Botrydiopsalean sp.	Botrydiopsalean	LH08AW1076	-
Botrydiopsis callosa (cf.)	Botrydiopsalean	LH08AW4043	-
Eustigmatos sp.	Eustigmatophyceae	LH10HG9133, LH10HG5036	-
Heterococcus caespitosus (cf.)	Chlorellidialean	LH08AG2020	-
Heterococcus chodatii (cf.)	Chlorellidialean	LH10HG9111	-
Heterococcus sp.	Chlorellidialean	LH10HG9085, LH10HG2140	-
Heterothrix sessile	Tribonematalean	LH10HG5079	-
Heterothrix sp.	Tribonematalean	LH10HG7061, LH08SG5052	-
Xanthonema bristolianum (cf.)	Tribonematalean	LH08HW9018	-
Xanthonema exile (cf.)	Tribonematalean	LH10HG7078	-
Xanthonema sp.	Tribonematalean	LH10HG9058, LH10HG7029	-

Table S3. List of the closest GenBank-relatives of our detected species.

Representative isolate(s) in 18S tree	Closest GenBank relatives; authentic strain*	GenBank	Similarity %	Habitat of origin	Land
LH10HG6096	*Auxenochlorella protothecoides* SAG 211-7a*	X56101	100.00	terrestrial/sap	DE
SAG 2478)	*Auxenochlorella protothecoides* SAG 211-7a*	X56101	97.46	terrestrial/sap	DE
LH08AG9040	*Chlorella mirabilis* SAG 38.88*	X74000	99.88	terrestrial/soil	RU
LH10HG6139	*Chlorella mirabilis* SAG 38.88*	X74000	99.82	terrestrial/soil	RU
LH08HG1081	*Chlorella vulgaris* SAG 211-11b*	FM205832	99.94	freshwater	NL
LH08SG3006	*Chlorella vulgaris* SAG 211-11b*	FM205832	100.00	freshwater	NL
LH10HG2081 (PS)	*Chlorella vulgaris* SAG 211-11b*	FM205832	99.75	freshwater	NL
LH10HG7072 (PS)	*Chlorella vulgaris* SAG 211-11b*	FM205832	99.82	freshwater	NL
LH10HG9105	*Chloroidium ellipsoideum* SAG 3.95*	FM946012	99.88	n/a	n/a
LH10HG7062	*Chloroidium saccharophilum* SAG 211-9a*	FM946000	100.00	terrestrial/sap	DE
SAG 2483	*Coccomyxa viridis* CCALA 306	AM167525	100.00	terrestrial/lichen	CZ
LH08SG9051 (PS)	*Coccomyxa simplex* SAG 216-8*	HQ317304	100.00	freshwater	RO
LH08AW1017	*Coccomyxa* sp. KN-2011-T3	HE586515	99.94	terrestrial/lichen	ID
LH08AW3050	*Dictyochloropsis splendida* CAUP H8601	GU017662	100.00	terrestrial/soil/fumarole	CZ
LH08HW8075	*Chlorella sphaerica* SAG 11.88*	AJ416105	99.94	terrestrial/lichen	NZ
LH08AG9089	*Chlorella sphaerica* SAG 11.88*	AJ416105	99.82	terrestrial/lichen	NZ
LH10HG3P15 (PS)	*Myrmecia bisecta* SAG 2043*	Z47209	100.00	terrestrial/soil	IT
LH08AW3064	*Myrmecia irregularis* CCAP 221/8	HQ902935	100.00	n/a	n/a
LH08SG3009	*Muriella terrestris* ASIB V38	AB012845	99.94	terrestrial/soil	IT
LH08SG8030	*Nannochloris bacillaris*	AB080300	99.94	n/a	n/a
SAG 2477)	*Leptochlorella* sp. clone QE17	FJ790649	95.54	terrestrial/epilithic	CN
LH10HG9080	*Neocystis brevis* CAUP D 802*	JQ920360	100.00	terrestrial/soil	CH
LH10HG3045	*Stichococcus mirabilis* CCAP 379/3	AJ311638	99.82	n/a	n/a
LH08SW8044	*Stichococcus mirabilis* CCAP 379/3	AJ311638	100.00	n/a	n/a
LH08AW8025	Trebouxiophyte sp. UR47/4	AY762604	100.00	terrestrial/facade	DE
LH08SG5057	Trebouxiophyte sp. UR55/3	AY762606	100.00	terrestrial/facade	DE
LH10HG6110, LH08AG7010	*Stichococcus deasonii* UTEX 1706*	DQ275460	99.70	terrestrial/soil	USA
LH08SG1073	*Stichococcus* sp.4 WB47	KF144240	100.00	freshwater/biofilm	DE
LH08SW1099	*Stichococcus deasonii* UTEX 1706*	DQ275460	98.90	terrestrial/soil	USA
SAG 2482	*Stichococcus* sp.2 D4-2A	KF144238	100.00	freshwater/biofilm	DE
SAG 2481	*Stichococcus* K4-4	AB055866	100.00	n/a	n/a
LH08AG1034	*Marvania* sp. WB67	KF144207	100.00	freshwater/biofilm	DE
LH10HG9020	*Nannochloris* sp. JL-4-6	AY195983	100.00	freshwater	USA
LH08SG3093	*Nannochloris* sp. Ant-1	EF440182	100.00	terrestrial/permafrost	AQ
LH10HG709K	*Nannochloris* sp. AS 2-10	AY195968	97.76	freshwater	USA
SAG 2382	*Xylochloris irregularis* CAUP H7801*	EU105209	96.47	terrestrial/epixylic	SG
LH08SG8041	*Scenedesmus rubescens* CCAP 232/1	X74002	100.00	n/a	n/a
LH10HG9034	*Bracteacoccus cohaerens* UTEX 1272*	GQ985406	100.00	terrestrial/soil	USA
LH08SG2015	*Bracteacoccus cohaerens* UTEX 1272*	GQ985406	99.94	terrestrial/soil	USA
LH08SW5031	*Chlamydomonas gerloffii* CCAP 11/72*	FR865610	99.82	freshwater	CZ
LH08SG1077	*Chlamydomonas rapa* SAG 48.72*	U70790	100.00	freshwater/plankton	SK
LH10HG1027	*Chlamydomonas rapa* SAG 48.72*	U70790	99.94	freshwater/plankton	SK
LH08SG9022 (PS)	*Chlamydomonas typica* SAG 61.72*	AB701557	99.92	terrestrial/soil	USA
LH10HG1013	*Chlorococcum robustum* Kr 86 30	AY122332	100.00	n/a	n/a

Table S3. (continuation)

Representative isolate(s) in 18S tree	Closest GenBank relatives; authentic strain*	GenBank	Similarity %	Habitat of origin	Land
SAG 2479	*Chlorococcum minutum* SAG 21.95	JN968585	99.77	terrestril/soil	IT
LH10HG3113	*Chlorococcum sphacosum* SAG 66.80*	JN968580	100.00	terrestrial/soil	USA
LH10HG7083	*Coelastrella multistriata* Hanagata C6-2	AB012846	100.00	terrestrial/epixylic	JP?
LH10HG2098 (+LH10HG7018)	*Scenedesmus* sp. KGU Y002	AB742453	100.00	n/a	n/a
LH08SG2049	*Desmotetra stigmatica* UTEX 962*	DQ009760	100.00	n/a	n/a
LH08AG2004	*Heterochlamydomonas rugosa* SAG 45.86	AF367859	99.76	freshwater	UK
SAG 2383)	*Jenufa minuta* CAUP H 8102*	HM563744	98.21	terrestrial/epixylic	SG
LH08SG8047	*Oogamochlamys gigantea* SAG 21.72*	AJ410468	98.41	terrestrial/soil	USA
SAG 2476 (PS)	*Chlamydomonas* sp. CCAP 11/159	FR865553	97.69	freshwater	USA
LH10HG2039	*Pseudomuriella aurantiaca* SAG 249-1*	X91268	100.00	terrestrial/soil	CH
LH10HG6108	Chlorococcales sp. VII3	FJ946904	99.59	freshwater	AQ
LH08SW7115	Uncultured Haematococcaceae clone Amb_18S_582	EF023273	99.77	terrestrial/soil	NL
LH10HG7016 (+LH10HG9131)	*Chlorococcum* cf. *tatrense* CCCryo 101-99	AF514407	99.71	n/a	n/a
LH08HW9058	*Pseudendocloniopsis botryoides* SAG 465-1*	AJ416103	99.88	freshwater	CH
LH08SG2033 (PS)	*Pedinomonas minor* SAG 1965-3	HE610132	99.80	freshwater/plankton	SK
LH08HW9106	*Klebsormidium dissectum* SAG 2155*	EF372518	100.00	terrestrial/soil	FR
LH10HG2056	*Klebsormidium flaccidum* SAG 7.91	EU434019	100.00	freshwater	RU
LH10HG3064	*Asterosiphon dichotomus* UTEX LB 2066	AM490829	98.54	n/a	n/a
LH08AW1076	*Chlorellidium pyrenoidosum* PAB 785	AJ579338	99.31	soil(?)	AQ
LH08AW4043	*Botrydiopsis callosa* SAG 30.83	AJ579340	99.29	soil	IT
LH10HG9133, LH10HG5036	*Eustigmatos magna* CCMP 387	U41051	99.82	terrestrial/soil	NZ
LH08AG2020	*Heterococcus protonematoides* SAG 835-9	AJ579334	99.94	terrestrial/soil	CH
LH10HG9111	*Heterococcus chodatii* SAG 835-3*	AM490822	100.00	terrestrial/subaerial	CH
LH10HG9085, LH10HG2140	Xanthophyceae sp. IX3	FJ946906	99.59	freshwater	AQ
LH10HG5079	*Heterothrix sessile* IBSG-V28	AM490818	100.00	n/a	n/a
LH10HG7061, LH08SG5052	*Heterothrix* sp.1 ACOL A1	AM491612	99.26-99.31	n/a	n/a
LH08HW9018	*Xanthonema bristolianum* CCALA 516	AM490819	100.00	terrestrial/snow	SK
LH10HG7078	*Xanthonema exile* PAB 395	AM491615	99.94	n/a	n/a
LH10HG9058, LH10HG7029	*Xanthonema exile* PAB 395	AM491615	99.89+99.77	n/a	n/a

Legend. AQ=Antarctica, CH=Switzerland, CN=China, CZ=Czech Republic, DE=Germany, FR=France, ID=Indonesia, IT=Italy, JP=Japan, NL=Netherlands, NZ=New Zealand, RO=Romania, RU=Russia, SG=Singapore, SK=Slovakia, UK=United Kingdom, USA=United States of America.

Table S4a. List of all analyzed full and partial (=PS) 18S rDNA sequences.

Species	Representative isolates (in phylogenetic trees)	Similar full 18S (OTU/0.00-level)	Similar partial 18S (OTU/0.00-level)
Auxenochlorella protothecoides	LH10HG6096	LH10HG7124, LH10HG5119	
Auxenochlorella sp.	LH08AW4103		
Chlorella mirabilis (cf.)	LH08AG9040		
Chlorella mirabilis (cf.)	LH10HG6139		
Chlorella vulgaris	LH08HG1081		
Chlorella vulgaris	LH08SG3006	LH10HG6014, LH08HG2065, LH08HG2013, LH08HG5074, LH08HW9060, LH10HG2049, LH08HG4088, LH10HG6052, LH10HG9075, LH08HW9094, LH08SG1071, LH08HG5082, LH08HG2083, LH08HG2091, LH08HG2096, LH08HG4032, LH10HG4093, LH10HG6099, LH10HG3088	LH08HW6087
Chlorella vulgaris (cf.)	LH10HG7072 (PS)		
Chlorella vulgaris (cf.)	LH10HG2081 (PS)		
Chloroidium ellipsoideum (cf.)	LH10HG9105	LH10HG6086, LH10HG7132, LH08AG8046, LH08AG9062	
Chloroidium saccharophilum	LH10HG7062	LH08AW8042	LH10HG5089
Coccomyxa viridis	SAG 2483		
Coccomyxa simplex	LH08SG9051 (PS)		
Coccomyxa sp.	LH08AW1017		
Dictyochloropsis splendida	LH08AW3050		
Diplosphaera sp.(I)	LH08HW8075		
Diplosphaera sp.(II)	LH08AG9089		
Lobosphaera bisecta (cf.)	LH10HG3P15 (PS)		
Lobosphaera irregularis (cf.)	LH08AW3064		LH08SW5063
Muriella terrestris (cf.)	LH08SG3009	LH10HG1070, LH10HG8050, LH10HG1076, LH10HG7118, LH10HG9077	
Nannochloris sp.	LH08SG8030	LH10HG6095	LH08SG3102
Navichloris fusiformis (proposed taxon)	SAG 2477		
Neocystis brevis	LH10HG9080	LH08SG2072, LH08AG9012, LH08AW8001, LH08HW6108, LH08HW6059, LH10HG4082, LH10HG9054	LH10HG9P02
Pseudostichococcus monallantoides	LH10HG3045	LH10HG2066	LH08AG7097
Pseudostichococcus sp.	LH08SW8044		
Stichococcus sp.(I)	LH08AW8025		LH08AG9028
Stichococcus sp.(II)	LH08SG5057	LH10HG7092, LH08SG5067, LH08SG5079, LH08SG5090, LH10HG2067, LH10HG2063, LH10HG9068, LH10HG3128, LH10HG7090	LH08AG1100, LH08SG5092
Stichococcus sp.(III)	LH10HG6110, LH08AG7010		
Stichococcus sp.(IV)	LH08SG1073		
Stichococcus sp.(V)	LH08SW1099		
Stichococcus sp.(VI)	SAG 2482	LH08AW8104	
Stichococcus sp.(VII)	SAG 2481		
Unidentified Chlorellaceae (I)	LH08AG1034		LH10HG7073
Unidentified Chlorellaceae (II)	LH10HG9020	LH08SG2053, LH10HG7071, LH10HG9026, LH10HG2094, LH10HG3135	LH08SG3029
Unidentified Chlorellaceae (III)	LH08SG3093	LH08SG3078	
Unidentified Chlorellaceae (IV)	LH10HG709K		
Xylochloris sp.	SAG 2382		
Acutodesmus rubescens	LH08SG8041		
Bracteacoccus cohaerens	LH10HG9034		
Bracteacoccus cohaerens (cf.)	LH08SG2015		
Chlamydomonas gerloffii (cf.)	LH08SW5031		
Chlamydomonas rapa	LH08SG1077	LH08SG9055	

Table S4a. (continuation)

Species	Representative isolates (in phylogenetic trees)	Similar full 18S (OTU/0.00-level)	Similar partial 18S (OTU/0.00-level)
Chlamydomonas rapa (cf.)	LH10HG1027		
Chlamydomonas typica (cf.)	LH08SG9022 (PS)		
Chlamydopodium vacuolatum	LH10HG1013		
Chlorococcum minutum (cf.)	SAG 2479	LH08AW5107	LH08AG701K, LH08AW5111
Chlorococcum sphacosum	LH10HG3113		
Coelastrella multistriata	LH10HG7083		LH10HG7102, LH10HG7100, LH10HG8109, LH10HG7097, LH08AG2003, LH08AW4118
Coelastrella sp.	LH10HG2098	LH10HG1009, LH10HG1033, LH10HG6060, LH10HG7017, LH10HG7023	LH10HG2087, LH10HG7018, LH10HG6035, LH10HG7030, LH10HG9130, LH10HG2P12, LH10HG2P01, LH10HG5136
Desmotetra stigmatica	LH08SG2049		LH10HG6P18
Heterochlamydomonas sp.	LH08AG2004		
Jenufa sp.	SAG 2383		LH08AW8098
Oogamochlamys sp.(I)	LH08SG8047		
*Oogamochlamys*sp.(II)	SAG 2476		
Pseudomuriella aurantiaca	LH10HG2039	LH10HG9038	
Stephanosphaerinia sp.	LH10HG6108		
Tatrensinia sp.(I)	LH08SW7115		
Tatrensinia sp.(II)	LH10HG7016		LH10HG9131
Pseudendocloniopsis botryoides (cf.)	LH08HW9058		
Pedinomonas minor (cf.)	LH08SG2033 (PS)		
Klebsormidium dissectum/elegans (cf.)	LH08HW9106	LH08AG9045, LH08HW9005, LH08AG1113, LH08HW1038, LH08AG7011, LH08HW4109, LH08HW5021	
Klebsormidium flaccidum (cf.)	LH10HG2056	LH08HW5061, LH10HG7028, LH08SG3027	
Asterosiphon sp.	LH10HG3064		
Botrydiopsalean sp.	LH08AW1076		LH08SG2K53
Botrydiopsis callosa (cf.)	LH08AW4043		
Heterococcus caespitosus (cf.)	LH08AG2020		
Heterococcus chodatii (cf.)	LH10HG9111		LH10HG9126
Heterococcus sp.	LH10HG9085, LH10HG2140		
Heterothrix sessile	LH10HG5079	LH10HG9037	
Heterothrix sp.	LH10HG7061, LH08SG5052		
Xanthonema bristolianum (cf.)	LH08HW9018	LH10HG6059	
Xanthonema exile (cf.)	LH10HG7078		
Xanthonema sp.	LH10HG9058, LH10HG7029	LH10HG3065, LH10HG9031, LH10HG1K69	LH10HG8112, LH10HW9129
Eustigmatos sp.	LH10HG9133, LH10HG5036		

Table S4b. Distribution of the green algal species across the sampling sites.

Species	Representative isolates (in phylogenetic trees)	Drill core (2008) SCH	Drill core (2008) ALB	Drill core (2008) HAI	Topsoil (2010) HAI	I	M	E
Auxenochlorella protothecoides	LH10HG6096				HEG5/6/7		1	1
Auxenochlorella sp.	LH08AW4103		AEW4				1	
Chlorella mirabilis (cf.)	LH08AG9040		AEG9					1
Chlorella mirabilis (cf.)	LH10HG6139				HEG6		1	
Chlorella vulgaris	LH08HG1081			HEG1		1		
Chlorella vulgaris	LH08SG3006	SEG1/3		HEG2/4/5, HEW6/9	HEG2/3/4/6/9	1	1	1
Chlorella vulgaris (cf.)	LH10HG7072 (PS)				HEG7			1
Chlorella vulgaris (cf.)	LH10HG2081 (PS)				HEG2	1		
Chloroidium ellipsoideum (cf.)	LH10HG9105		AEG8/9		HEG6/7/9			1
Chloroidium saccharophilum	LH10HG7062		AEW8		HEG5/7		1	1
Coccomyxa viridis	SAG 2483		AEW8					1
Coccomyxa simplex	LH08SG9051 (PS)	SEG9						1
Coccomyxa sp.	LH08AW1017		AEW1			1		
Dictyochloropsis splendida	LH08AW3050		AEW3			1		
Diplosphaera sp.(I)	LH08HW8075			HEW8				1
Diplosphaera sp.(II)	LH08AG9089		AEG9					1
Lobosphaera bisecta (cf.)	LH10HG3P15 (PS)				HEG3	1		
Lobosphaera irregularis (cf.)	LH08AW3064	SEW5	AEW3			1	1	
Muriella terrestris (cf.)	LH08SG3009	SEG3			HEG1/7/8/9	1		1
Nannochloris sp.	LH08SG8030	SEG3/8			HEG6	1	1	1
Navichloris fusiformis (proposed taxon)	SAG 2477		AEW3			1		
Neocystis brevis	LH10HG9080	SEG2	AEG9, AEW8	HEW6	HEG4/9	1	1	1
Pseudostichococcus monallantoides	LH10HG3045		AEG7		HEG2/3	1		1
Pseudostichococcus sp.	LH08SW8044	SEW8						1
Stichococcus sp.(I)	LH08AW8025		AEG9/AEW8					1
Stichococcus sp.(II)	LH08SG5057	SEG5	AEG1		HEG2/3/7/9	1	1	1
Stichococcus sp.(III)	LH10HG6110, LH08AG7010		AEG7		HEG6		1	1
Stichococcus sp.(IV)	LH08SG1073	SEG1				1		
Stichococcus sp.(V)	LH08SW1099	SEW1				1		
Stichococcus sp.(VI)	SAG 2482		AEW8					1
Stichococcus sp.(VII)	SAG 2481		AEW8					1
Unidentified Chlorellaceae (I)	LH08AG1034		AEG1		HEG7	1		1
Unidentified Chlorellaceae (II)	LH10HG9020	SEG2/3			HEG2/3/7/9	1		1
Unidentified Chlorellaceae (III)	LH08SG3093	SEG3				1		
Unidentified Chlorellaceae (IV)	LH10HG709K				HEG7			1
Xylochloris sp.	SAG 2382		AEG7					1
Acutodesmus rubescens	LH08SG8041	SEG8						1
Bracteacoccus cohaerens	LH10HG9034				HEG9			1
Bracteacoccus cohaerens (cf.)	LH08SG2015	SEG2				1		
Chlamydomonas gerloffii (cf.)	LH08SW5031	SEW5					1	
Chlamydomonas rapa	LH08SG1077	SEG1/9				1		1
Chlamydomonas rapa (cf.)	LH10HG1027				HEG1	1		
Chlamydomonas typica (cf.)	LH08SG9022 (PS)	SEG9						1
Chlamydopodium vacuolatum	LH10HG1013				HEG1	1		
Chlorococcum minutum (cf.)	SAG 2479		AEG7/AEW5				1	1
Chlorococcum sphacosum	LH10HG3113				HEG3	1		
Coelastrella multistriata	LH10HG7083		AEG2/AEW4		HEG7/8	1	1	1
Coelastrella sp.	LH10HG2098				HEG1/2/5/6/7/9	1	1	1
Desmotetra stigmatica	LH08SG2049	SEG2			HEG6	1	1	
Heterochlamydomonas sp.	LH08AG2004		AEG2			1		
Jenufa sp.	SAG 2383		AEW8					1
Oogamochlamys sp.(I)	LH08SG8047	SEG8						1
Oogamochlamys sp.(II)	SAG 2476		AEW1			1		

Table S4b. (continuation)

Species	Representative isolates (in phylogenetic trees)	Drill core (2008) SCH	Drill core (2008) ALB	Drill core (2008) HAI	Topsoil (2010) HAI	I	M	E
Pseudomuriella aurantiaca	LH10HG2039				HEG2/9	1	0	1
Stephanosphaerinia sp.	LH10HG6108				HEG6		1	
Tatrensinia sp.(I)	LH08SW7115	SEW7						1
Tatrensinia sp.(II)	LH10HG7016				HEG7/9			1
Pseudendocloniopsis botryoides (cf.)	LH08HW9058			HEW9				1
Pedinomonas minor (cf.)	LH08SG2033 (PS)	SEG2				1		
Klebsormidium dissectum/elegans (cf.)	LH08HW9106		AEG1/7/9	HEW1/4/5/9		1	1	1
Klebsormidium flaccidum (cf.)	LH10HG2056	SEG3		HEW5	HEG2/7	1	1	1
Asterosiphon sp.	LH10HG3064				HEG3	1		
Botrydiopsalean sp.	LH08AW1076	SEG2	AEW1			1		
Botrydiopsis callosa (cf.)	LH08AW4043		AEW4				1	
Heterococcus caespitosus (cf.)	LH08AG2020		AEG2			1		
Heterococcus chodatii (cf.)	LH10HG9111				HEG9			1
Heterococcus sp.	LH10HG9085, LH10HG2140				HEG2/9	1		1
Heterothrix sessile	LH10HG5079				HEG5/9		1	1
Heterothrix sp.	LH10HG7061, LH08SG5052	SEG5			HEG7			1
Xanthonema bristolianum (cf.)	LH08HW9018			HEW9	HEG6		1	
Xanthonema exile (cf.)	LH10HG7078				HEG7			1
Xanthonema sp.	LH10HG9058, LH10HG7029				HEG1/3/7/8/9, HEW9	1		1
Eustigmatos sp.	LH10HG9133, LH10HG5036				HEG5/9		1	1

Legend. PS=partial sequence; Land-use intensity: E=extensive, M=managed, I=intensive; Exploratory: ALB=Schwäbische Alb, HAI=Hainich-Dün, SCH=Schorfheide-Chorin.

Table S5. General morphological characteristics of the new isolates.

Representative isolates	Morphotype assignation	Cell shape	Cell size	Figure
LH10HG6096	*Chlorella*	spherical	Ø=2.9-6.5 µm	Figure 7k
SAG 2478	*Chlorella*	spherical	Ø=2.2-5.2 µm	Figure 7l
LH08AG9040	*Chlorella*-like	spherical	Ø=3.0-6.4 µm	Figure 7a
LH10HG6139	*Chlorella*-like	spherical	n/a	n/a
LH08HG1081	*Chlorella*	spherical/ellipsoidal	Ø=2.3-5.2 µm	Figure 7b
LH08SG3006	*Chlorella*	spherical/ellipsoidal	n/a	n/a
LH10HG2081 (PS)	*Chlorella*	spherical/ellipsoidal	Ø=2.5-6.2 µm	Figure 7c
LH10HG7072 (PS)	*Chlorella*	spherical/ellipsoidal	n/a	n/a
LH10HG9105	*Chloroidium*	ellitpical	l=4.8-7.8 µm, w=3.3-5.3 µm	Figure 6i
LH10HG7062	*Chloroidium*	ellitpical	l=5.6-9.1 µm, w=4.0-6.2 µm	Figure 6h
SAG 2483	*Coccomyxa*	fusiform (asymmetrical)	l=4.7-8.4 µm, w=1.8-3.6 µm	Figure 6f
LH08SG9051 (PS)	*Coccomyxa*	fusiform (asymmetrical)	l=4.4-7.8 µm, w=2.0-4.3 µm	Figure 6e
LH08AW1017	*Coccomyxa*	fusiform (asymmetrical)	n/a	n/a
LH08AW3050	*Dictyochloropsis*	spherical	Ø=7.7-39.4 µm	Figure 6l
LH08HW8075	*Diplosphaera*	broadly oval/cylindric	l=3.2-6.2 µm, w=1.9-4.4 µm	Figure 8b (Chapter 3)
LH08AG9089	*Diplosphaera*	broadly oval/cylindric	n/a	n/a
LH10HG3P15 (PS)	*Lobosphaera*	spherical	Ø=6.2-9.6 µm	Figure 6j
LH08AW3064	*Lobosphaera*	spherical	n/a	n/a
LH08SG3009	*Chlorella*	spherical	Ø=1.7-4.2 µm	Figure 3b (Chapter 3)
LH08SG8030	*Chlorella*	spherical	Ø=2.2-3.9 µm	Figure 7e (Chapter 3)
SAG 2477	*Coccomyxa*-like	fusiform (symmetrical)	l=8.3-15.3 µm, w=3.6-7.8 µm	Figure 6g
LH10HG9080	*Neocystis*	broadly oval	l=4.8-8.7 µm, w=2.7-6.6 µm	Figure 6d
LH10HG3045	*Stichococcus*	cylindrical	n/a	n/a
LH08SW8044	*Stichococcus*	cylindrical	l=3.4-9.8 µm; w=1.6-3.3 µm	Figure 8d (Chapter 3)
LH08AW8025	*Stichococcus*	cylindrical	l=1.7-19.8 µm, w=1.4-3.6 µm	Figure 6a
LH08SG5057	*Stichococcus*	cylindrical	l=4.5-9.8 µm, w=1.9-3.3 µm	Figure 6b
LH10HG6110, LH08AG7010	*Stichococcus*	cylindrical	n/a	n/a
LH08SG1073	*Stichococcus*	cylindrical	n/a	n/a
LH08SW1099	*Stichococcus*/ *Diplosphaera*	cylindrical/packages	l=3.0-6.3 µm; w=1.0-4.2 µm	Figure 8j (Chapter 3)
SAG 2482	*Stichococcus*	cylindrical	l=3.8-7.2 µm, w=1.9-6.3	Figure 6c
SAG 2481	*Stichococcus*	cylindrical	l=3.1-8.7 µm; w=1.6-2.9 µm	Figure 8e (Chapter 3)
LH08AG1034	*Chlorella*	spherical	Ø=2.4-4.5 µm	Figure 7g
LH10HG9020	*Chlorella*	spherical	Ø=2.4-4.8 µm	Figure 7h
LH08SG3093	*Chlorella*	spherical	Ø=2.3-3.8 µm	Figure 3d (Chapter 3)
LH10HG709K	*Chlorella*	spherical/ellipsoidal	l=3.1-5.1 µm, w=2.0-3.7 µm	Figure 7j
SAG 2382	*Trebouxia*-like	spherical	l=4.7-12.9 µm, w=3.1-10.6 µm	Figure 6k
LH08SG8041	*Scenedesmus*-like	ellipsoidal, obtuse cell poles	Ø=3.9-7.4 µm	Figure 3g
LH10HG9034	*Bracteacoccus*-like	spherical	Ø=5.4-12.3 µm	Figure 3i
LH08SG2015	*Bracteacoccus*-like	spherical	n/a	n/a
LH08SW5031	*Chlamydomonas*	oval flagellated	l=10.9-16.4 µm, w=4.3-10.1 µm	Figure 4c
LH08SG1077	*Chlamydomonas*	oval flagellated	n/a	n/a
LH10HG1027	*Chlamydomonas*	oval flagellated	l=6.1-9.9 µm, w=2.7-5.1 µm	Figure 4d
LH08SG9022 (PS)	*Chlamydomonas*	oval flagellated	l=6.7-12.2 µm, w=4.0-8.0 µm	Figure 4f

Table S5. (continuation)

Representative isolates	Morphotype assignation	Cell shape	Cell size	Figure
LH10HG1013	*Chlorococcum*	spherical/oval	n/a	n/a
SAG 2479	*Chlorococcum*	spherial/oval	l=5.6-9.5 µm, w=5.6-9.5 µm	Figure 4k
LH10HG3113	*Chlorococcum*	spherial/oval	l=5.8-9.3 µm, w=2.6-6.3 µm	Figure 4j
LH10HG7083	*Scenedesmus*-like	ellipsoidal, obtuse cell poles	Ø=3.5-7.0 µm	Figure 3e
LH10HG2098 LH10HG7018	*Scenedesmus*-like	ellipsoidal, obtuse cell poles	l=6.8-12.5 µm, w=7.0-10.9 µm	n/a Figure 3f
LH08SG2049	*Chlorosarcina*-like	spherial	Ø=3.5-6.6 µm	Figure 4g
LH08AG2004	*Chlamydomonas*	oval flagellated	l=6.0-10.5 µm, w=3.1-7.5 µm	Figure 4e
LH08AW8035 (= SAG 2383)	*Bracteacoccus*-like	spherical	Ø=2.9-7.3 µm	Figure 4l
LH08SG8047	*Chlamydomonas*	oval flagellated	Ø=5.1-8.9 µm	Figure 4a
SAG 2476 (PS)	*Chlamydomonas*	oval flagellated	l=9.1-14.5 µm, w=5.1-10.0 µm	Figure 4b
LH10HG2039	*Bracteacoccus*-like	spherical	Ø=3.6-8.2 µm	Figure 3h
LH10HG6108	*Chlorococcum*	spherical/oval	n/a	n/a
LH08SW7115	*Chlorococcum*	spherical/oval	l=8.3-17.8 µm, w=5.2-13.3 µm	Figure 4h
LH10HG7016 LH10HG913	*Chlorococcum*	spherical/oval	Ø=7.9-13.1 µm	n/a Figure 4i
LH08HW9058	*Pseudendocloniopsis*	sphaerical, obtuse cell pole	Ø=4.7-9.5 µm	Figure 3d
LH08SG2033 (PS)	*Chlamydomonas*-like	oval flagellated	l=2.9-5.6 µm, w=2.5-4.8 µm	Figure 3c
LH08HW9106	*Klebsormidium*	trichal	l=4.2-12.8 µm, w=4.1-6.2 µm	Figure 3b
LH10HG2056	*Klebsormidium*	trichal	l=6.4-19.7 µm, w=4.5-7.0 µm	Figure 3a
LH10HG3064	*Asterosiphon*	spherical/siphonal	Ø=5.4-12.1 µm	Figure 11k
LH08AW1076	*Botrydiopsis*	sphaerical	Ø=4.9-10.2 µm	Figure 11i
LH08AW4043	*Botrydiopsis*	sphaerical	Ø=5.1-8.8 µm	Figure 11j
LH10HG9133, LH10HG5036	*Eustigmatos*	spherical	Ø=6.1-13.1 µm	Figure 11l n/a
LH08AG2020	*Heterococcus*	heterotrichal	n/a	n/a
LH10HG9111	*Heterococcus*	heterotrichal	l=5.2-12.4 µm, w=4.1-13.2 µm	Figure 11g, h
LH10HG9085, LH10HG2140	*Heterococcus*	heterotrichal	n/a	Figure 11f
LH10HG5079	*Xanthonema*	trichal	n/a	n/a
LH10HG7061, LH08SG5052	*Xanthonema*	trichal	l=10.2-25.4 µm, w=2.7-10.7 µm	Figure 11d Figure 11e
LH08HW9018	*Xanthonema*	trichal	l=3.3-15.3 µm, w=2.9-5.7 µm	Figure 11c
LH10HG7078	*Xanthonema*	trichal	l=4.2-11.2 µm, w=3.3-6.0 µm	Figure 11b
LH10HG9058, LH10HG7029	*Xanthonema*	trichal	l=5.7-12.1 µm, w=3.4-6.5 µm	Figure 11a, n/a

Legend. PS=partial sequence.

Table S6a. Diversity of morphospecies in soil drill cores.

Soil horizon	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h	A-h
Exploratory	H	H	H	H	H	H	A	A	A	A	A	A	A	S	S	S	S	S	S
Landscape/plot Nr.	G/1-3	G/4-6	G/7-9	W/1-3	W/4-6	W/7-12	G/1-3	G/4-6	G/7-9	W/1-3	W/4-6	W/7-9	G/1-3	G/4-6	G/7-9	W/1-3	W/4-6	W/7-9
Land-use	E	M	I	E	M	I	E	M	I	E	M	I	E	M	I	E	M	I
Subsample	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX	MX
Bracteacoccus	-	-	-	1	1	-	1	1	-	-	-	-	1	1	-	-	-	-
Characiochloridaceae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlamydomonas	1	1	-	-	1	1	-	-	1	1	-	1	1	1	1	-	1	1
Chlorococcum	1	1	1	-	1	1	-	1	-	1	1	1	1	1	1	-	1	-
Chlorosarcinaceae	-	-	1	-	-	-	1	-	1	-	-	-	-	1	-	-	-	-
Monoraphidium terrestre	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Radiococcaceae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scenedesmaceae	-	1	-	-	1	-	1	-	1	-	1	-	1	1	1	-	-	-
Chlorella	1	1	1	1	1	1	1	1	1	1	1	-	1	1	1	1	1	-
Chloroidium	-	-	-	-	-	1	-	-	-	-	1	-	-	1	-	-	-	-
Coccomyxa	-	1	-	1	1	1	-	-	-	1	-	1	1	1	1	-	-	-
Dictyochloropsis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Keratococcus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lobosphaera	-	-	1	-	1	1	1	-	-	1	-	1	1	-	1	-	-	1
Muriella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neocystis	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stichococcus	-	1	-	1	1	1	1	-	1	1	1	1	1	1	1	1	1	1
Dilabifilum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kentrosphaera	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudendocloniopsis	1	1	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-	-
Pseudendoclonium	-	-	-	-	-	1	-	-	1	-	1	1	-	-	-	-	-	-
Cosmarium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsormidium	-	-	-	-	1	1	1	-	1	-	-	1	1	1	-	1	-	1
Porphyridium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diadesmis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hantzschia	1	1	1	-	1	-	-	-	-	-	-	-	1	-	1	-	-	-
Mayamaea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Navicula	1	1	1	-	1	-	-	1	-	-	-	-	1	1	1	-	-	-
Nitzschia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinnularia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stauroneis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Surirella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eustigmatos	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Heterococcus	-	-	-	-	1	-	1	-	-	1	-	1	1	1	-	-	-	-
Tribonema	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xanthonema	-	-	-	-	-	1	1	-	-	1	-	-	-	-	-	-	-	-
Xanth. coccal	1	1	1	-	1	-	1	-	1	-	-	1	1	1	-	1	-	-
Cryptomonas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cylindrospermum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptolyngbya	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nostoc	1	1	-	-	1	-	-	1	1	1	-	-	-	1	1	-	-	-
Phormidium	1	1	1	-	1	-	1	-	1	1	-	-	1	1	1	-	-	-
Plectonema	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudanabaena	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stigonema	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Legend. A-h=drill core; O-h=topsoil. H=Hainich-Dün; A=Schwäbische Alb; S=Schorfheide-Chorin. G=grassland; W=forest. E=extensive; M=managed; I=intensive. MX=mixed.

Table S6b. Diversity of morphospecies in topsoil samples.

Soil horizon	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h	O-h
Exploratory	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
Landscape/plot Nr.	G/7	G/7	G/7	G/7	G/8	G/8	G/8	G/8	G/9	G/9	G/9	G/9	W/5	W/5	W/5	W/5	W/6	W/6	W/6	W/6
Land-use	E	E	E	E	E	E	E	E	E	E	E	E	M	M	M	M	M	M	M	M
Subsample	MD	NE	SW	MX	MD	NE	SW	MX	MD	NE	SW	MX	MD	NE	SW	MX	MD	NE	SW	MX
Bracteacoccus	-	1	-	1	-	-	1	-	1	-	1	1	-	-	-	-	-	-	-	-
Characiochloridaceae	-	1	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-	-	1	-
Chlamydomonas	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorococcum	1	1	1	1	1	1	1	1	1	1	1	1	-	1	1	1	-	1	1	1
Chlorosarcinaceae	-	-	-	1	-	-	-	-	1	1	-	1	-	-	-	-	-	-	-	-
Monoraphidium terrestre	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-
Radiococcaceae	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scenedesmaceae	1	1	1	1	1	1	1	1	1	1	1	1	-	1	-	-	-	-	-	-
Chlorella	-	1	1	-	1	1	-	1	1	1	1	-	-	-	-	-	-	-	-	-
Chloroidium	-	-	-	-	1	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-
Coccomyxa	-	-	-	1	1	1	1	1	1	1	1	1	1	1	1	1	-	1	1	-
Dictyochloropsis	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Keratococcus	1	1	-	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-
Lobosphaera	-	1	-	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	1
Muriella	1	-	-	1	-	1	1	-	1	1	1	1	-	1	-	-	-	-	-	-
Neocystis	-	-	-	-	1	-	-	-	1	-	1	-	-	-	-	-	-	-	1	-
Stichococcus	1	1	-	1	1	1	1	1	1	1	1	1	-	-	1	1	1	-	1	-
Dilabifilum	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Kentrosphaera	-	-	-	-	-	-	-	1	-	1	1	1	-	-	-	-	-	-	-	-
Pseudendocloniopsis	1	1	1	1	1	1	1	-	1	1	1	1	-	-	-	-	-	-	-	-
Pseudendoclonium	-	-	-	-	-	-	1	-	1	1	1	1	-	-	-	-	-	-	-	-
Cosmarium	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Klebsormidium	-	1	1	1	-	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-
Porphyridium	-	-	-	-	-	-	-	-	-	1	1	1	-	-	-	-	-	-	-	-
Diadesmis	-	1	-	1	-	-	1	-	1	-	1	-	-	-	-	-	-	-	-	-
Hantzschia	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-
Mayamaea	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	1
Navicula	-	-	1	-	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Nitzschia	-	-	-	-	1	1	-	1	-	1	1	1	-	-	-	-	-	-	-	-
Pinnularia	-	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-	-	-	-
Stauroneis	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Surirella	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-
Eustigmatos	-	1	-	-	-	-	1	1	1	1	1	1	-	-	-	1	-	-	-	-
Heterococcus	1	1	-	1	1	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tribonema	1	-	-	-	1	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-
Xanthonema	1	1	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-	-	1
Xanth. coccal	1	1	-	1	1	-	1	1	1	1	1	1	-	-	-	1	1	-	-	-
Cryptomonas	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Cylindrospermum	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Leptolyngbya	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-
Nostoc	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-
Phormidium	1	-	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-
Plectonema	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Pseudanabaena	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Stigonema	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-

Legend. A-h=drill core; O-h=topsoil. H=Hainich-Dün; A=Schwäbische Alb; S=Schorfheide-Chorin. G=grassland; W=forest. E=extensive; M=managed; I=intensive. MX=mixed; MD=middle; NE=NorthEast; SW=SouthWest.

Table S7. Frequency of the morphospecies in soil drill cores.

Morphotype and the authority	Nr. obs	% of samples	Microphotographs	Isolate/mixed culture
Bracteacoccus Tereg	12	32	Figure 3i	isolate
Characiochloridaceae Skuja	6	16	Supplementary Figure 1a-b	mixed culture
Chlamydomonas Ehrenberg	32	84	Figure 4a-f	isolate
Chlorococcum Meneghini	31	82	Figure 4h-k	isolate
Chlorosarcinaceae Bourrelly ex Groover & Bold	8	21	Supplementary Figure 1f	isolate
Monoraphidium terrestre (Bristol) Krienitz	2	5	Supplementary Figure 1h	mixed culture
Radiococcaceae Fott & Komárek	1	3	n/a	mixed culture
Scenedesmaceae Oltmanns	21	55	Supplementary Figure 1g	isolate
Chlorella Beijerinck	24	63	Figure 7a-c	isolate
Chloroidium Nadson	6	16	Figure 6h-i	isolate
Coccomyxa Schmidle	24	63	Figure 6e-g	isolate
Dictyochloropsis Geitler em. Tschermak-Woess	1	3	Figure 6l	isolate
Keratococcus Pascher	11	29	Supplementary Figure 1i-j	mixed culture
Lobosphaera Reisigl	20	53	Figure 6j	isolate
Muriella J. B. Petersen	9	24	Figure 7d-i	isolate
Neocystis F. Hindák	6	16	Figure 6d	isolate
Stichococcus Nägeli	30	79	Figure 6a-c	isolate
Dilabifilum Tschermak-Woess	1	3	Supplementary Figure 2b	mixed culture
Kentrosphaera Borzì	4	11	Supplementary Figure 1l	mixed culture
Pseudendocloniopsis Vischer	15	39	Figure 3d	isolate
Pseudendoclonium Wille	9	24	Supplementary Figure 2a	isolate
Cosmarium Corda	1	3	Supplementary Figure 2d-e	mixed culture
Klebsormidium Silva, Mattox 8c Blackwell	23	61	Figure 3a-b	isolate
Porphyridium Nägeli	3	8	Supplementary Figure 2h	mixed culture
Diadesmis contenta (Grunow) D. G. Mann	5	13	Supplementary Figure 3b	mixed culture
Hantzschia amphioxys (Ehrenberg) Grunow	18	47	Supplementary Figure 3o	mixed culture
Mayamaea H. Lange-Bertalot	13	34	Supplementary Figure 3e	mixed culture
Navicula Bory de Saint-Vincent	24	63	Supplementary Figure 3a,c-d	mixed culture
Nitzschia Hassall	6	16	Supplementary Figure 3m-n	mixed culture
Pinnularia Ehrenberg	4	11	Supplementary Figure 3f-i	mixed culture
Stauroneis Ehrenberg	1	3	Supplementary Figure 3j	mixed culture
Surirella Turpin	2	5	Supplementary Figure 3l	mixed culture
Eustigmatos D. J. Hibberd	10	26	Figure 11l	isolate
Heterococcus Chodat	24	63	Figure 11f-h	isolate
Tribonema Derbès & Solier	4	11	Supplementary Figure 2j	mixed culture
Xanthonema Silva	20	53	Figure 11a-e	isolate
Xanth. coccal	22	58	Figure 11i-j; Suppl. Figure 2k-l	Isolate; mix.cul.
Cryptomonas Ehrenberg	1	3	Supplementary Figure 2i	mixed culture
Cylindrospermum F. T. Kützing ex É. Bornet & C. Flahault	2	5	Supplementary Figure 4j	mixed culture
Leptolyngbya Anagnostidis & Komárek	12	32	Supplementary Figure 4b-e	mixed culture
Nostoc Vaucher ex Bornet & Flahault	20	53	Supplementary Figure 4g-i	mixed culture
Phormidium Kützing ex Gomont	21	55	Supplementary Figure 4f	mixed culture
Plectonema Thuret ex Gomont	1	3	n/a	mixed culture
Pseudanabaena Lauterborn	1	3	Supplementary Figure 4a	mixed culture
Stigonema Agardh ex Bornet et Flahault	1	3	Supplementary Figure 4l	mixed culture

Appendix | Chapter 2

Supporting Tables

Table S1. Species of green algae recovered from creek biofilms.

Table S2. Green algal species recovered from creek biofilms and their main morphological features.

Table S3. Percentage identities (PID) to closest GenBank-relatives.

Table S4. Sequences representing additional groups and lineages shown as collapsed triangles in **Figures 1-3**.

Table S1. Species of green algae recovered from creek biofilms.

Species	Isolate(s) studied in detail	Sequence accession numbers (original identifiers)	Additional isolates	Sequence accession numbers
Chlorella sp.	RK52	KF144182	DB1-5, DB1-10	KF144171, KF144173
	D11-2	KF144172	DB2-4, DB2-7	KF144177-KF144181
			DB3-2, DB6-28	KF144174-KF144176
			DB6-33, DB14-17	
			DB14-21, DB14-23	
Coccomyxa cf. *pringsheimii*	WB28	KF144225	DB14-12, SAG 2391	KF144223
	WB32			KF144224 (DB14-14)
Coccomyxa cf. *simplex*	WB40	KF144226		
Elliptochloris subsphaerica	WB5-D1e	KF144205	WB27	KF144204
Marvania sp.	WB67	KF144207		
Muriella terrestris	D6-DB2	KF144209	SAG 2390	
Neocystis cf. *mucosa*	SAG 2405	KF144212 (WB21)		
Stichococcus bacillaris	WB13	KF144231		
	WB74	KF144232		
Stichococcus cf. *deasonii*	WB38	KF144233		
Stichococcus mirabilis	WB69	KF144234		
Stichococcus sp.1	DB6-27	KF144235		
	WB65	KF144236		
	SAG 2407	KF144237 (WB68)		
Stichococcus sp.2	D4-2A	KF144238		
Stichococcus sp.3	SAG 2408	KF144239 (WB8)		
Stichococcus sp.4	SAG 2406	KF144240 (WB47)		
Acutodesmus obliquus	D22-6-2B	KF144164		
Bracteacoccus aerius-relative	SAG 2403	KF144165 (WB18)		
Bracteacoccus sp.	DB9-3	KF144166		
Chlamydomonas sp.	RK68	KF144168	DB6-21	KF144167
Chlamydopodium sp.	SAG 2402	KF144169 (RK41)		
	RK50	KF144170		
Chlorococcum sphacosum	SAG 2398	KF144183 (GRK6-DB1)		
Chlorococcum ellipsoideum-relatives1	GRK6-DB5	KF144184		
Chlorococcum ellipsoideum-relatives2	SAG 2400	KF144186 (GRK7-WB4)		
	GRK6-DB6	KF144185		
	SAG 2401	KF144187 (GRK7-WB5)		
Chlorococcum minutum-relative	SAG 2399	KF144188 (GRK6-DB3)		
Desmodesmus cf. *armatus*	RK43	KF144195	DB6-23, DB6-26	KF144192-KF144194
	SAG 2395	KF144191 (D7-6-1B)	DB6-30, SAG 2389	
Monoraphidium terrestre cf. *dybowskii*	SAG 2393	KF144208 (D21-6-5B)		
Mychonastes cf. *homosphaera*	RK48	KF144210		
Mychonastes sp.	DB6-29	KF144211		
Pseudomuriella cf. *schumacherensis*	RK3	KF144227		
Scenedesmaceae sp.	RK49	KF144228		
Desmochloris cf. *halophila*	SAG 2397	KF144190 (DB1-9)		
Dilabifilum printzii	WB41	KF144201	DB15-6, WB3	KF144197, KF144200
			WB24, WB31	KF144199, KF144196
			WB43, WB59	KF144202, KF144203
Hazenia mirabilis	SAG 2396	KF144206 (D9-14B)		
Pseudendocloniopsis botryoides	SAG 2394	KF144213 (D2-6-1A)	DB14-6	KF144218
	DB6-19	KF144219	DB14-11	KF144214-KF144217
			DB14-24	KF144221
			DB14-28	KF144220
			DB14-45	
			WB4	
			WB45	
Pseudendoclonium akinetum	SAG 2404	KF144222 (WB20)		

Legend. The 74 unialgal isolates, their affiliation to a certain class, their strain designations and NCBI accession numbers of their 18S rRNA gene sequences are listed. The 45 strains used as exemplar for detailed examination are listed separately from those additional strains representing the same species. When an isolate was accessioned by the SAG culture collection, the corresponding SAG strain number is given and the original strain designation is in brackets next to its sequence accession number.

Table S2. Green algal species recovered from creek biofilms and their main morphological features.

Species	Figure	Morphological features
Chlorella sp.	Figure 5c, 5d	Cells globose or ellipsoidal with smooth cell walls. Single parietal chloroplast with single pyrenoid. Reproduction by 2-8 autospores per cell, released by rupture of parental cell wall. Cell diameter 4.5-8.0 µm.
Coccomyxa cf. *pringsheimii*	Figure 5i	Heteropolar cells oval, fusiform or egg-shaped with smooth cell walls. Single parietal chloroplast without pyrenoids. Cell diameter: 4.4-8.0 µm.
Coccomyxa cf. *simplex*	n/a	Heteropolar cells oval, fusiform or egg-shaped with smooth cell walls. Single parietal chloroplast without pyrenoids. Cell length: 4.6-8.3 µm.
Elliptochloris subsphaerica	Figure 5h	Young cells cylindric, slightly inflexed, mature cells ellipsoidal to globose. Single parietal chloroplast with single pyrenoid. Autosporangia ellipsoidal with several cylindric autospores. Cell diameter 7.0-10.5 µm.
Marvania sp.	Figure 5f	Cells spherical, with 2 parietal chloroplasts, pyrenoids absent. Cell diameter 2.5-4.2 µm.
Muriella terrestris	Figure 5e	Cells spherical, with 2 or more parietal chloroplasts without a pyrenoid. Asexual reproduction by autospores, released by rupture of parental cell wall. Cell diameter: 2.4-4.4 µm.
Neocystis cf. *mucosa*	Figure 5g	Cells broadly oval with single parietal chloroplast without a pyrenoid. Asexual reproduction by autospores, 2-4 per autosporangium, released by rupture of the cell wall, remaining visible in the colony. Cell diameter: 2.9-5.7 µm.
Stichococcus bacillaris	Figure 5a	Few-celled fragmenting filaments or individual cells. Cells cylindrical or elongate, sometimes slightly oval with single parietal chloroplast without pyrenoids. Cell length: 4.1-17.4 µm.
Stichococcus cf. *deasonii*	n/a	Cylindrical elongate cells with a single parietal chloroplast containing a single starch-enveloped pyrenoid, cell length: 4.1-8.9 µm.
Stichococcus mirabilis	n/a	For description see *Stichococcus bacillaris* above, cell length: 3.2-9.5 µm.
Stichococcus sp.1	n/a	For description see *Stichococcus bacillaris* above, cell length: 2.9-11.9 µm.
Stichococcus sp.2	n/a	For description see *Stichococcus* cf. *deasonii* above, cell length: 6.2-11.4 µm.
Stichococcus sp.3	n/a	For description see *Stichococcus* cf. *deasonii* above, cells sometimes flexed. Cell length: 6.2-11.4 µm.
Stichococcus sp.4	Figure 5b	For description see *Stichococcus* cf. *deasonii* above, cell length: 3.7-9.5 µm.
Acutodesmus obliquus	Figure 6h	Fusiform cells with acute cell poles, without bristles. Cell length: 6.2-10.9 µm.
Bracteacoccus sp.	Figure 7b	Sphaerical cells with several to many parietal lens-shaped chloroplasts without pyrenoids. Cell diameter: 4.9-10.0 µm.
Chlamydomonas sp.	Figure 6f	Spherical cells with anterior contractile vacuole and two isokont anterior flagella. Cells contain single cup-shaped chloroplast with one basal pyrenoid and an eyespot at the cell anterior. Cell length: 7.2-11.1 µm.
Chlorococcum cf. *sphacosum*	Figure 6a	Cells spherical to ellipsoidal with smooth cell walls. One parietal chloroplast per cell containing single pyrenoid. Biflagellated zoospores remain ellipsoidal after motility ceases (*Chlamydomonas*-type). Cell diameter: 7.0-10.5 µm.
Chlorococcum ellipsoideum-relatives1	Figure 6c	Cells spherical to ellipsoidal with smooth cell walls. One parietal chloroplast per cell containing single pyrenoid. Cell diameter: 8.5-11.7 µm.
Chlorococcum ellipsoideum-relatives2	Figure 6d, 6e	Spherical cells with anterior contractile vacuole, possessing two isokont anterior flagella. Single band-shaped chloroplast per cell with a single lateral pyrenoid and an eyespot at the cell anterior. Cell length: 5.8-11.8 µm.
Desmodesmus cf. *armatus*	Figure 6g	Ellipsoidal cells with obtuse cell poles and bristles. Cells form 4-8-celled coenobia. Cell length: 6.1-17.6 µm.
Mychonastes cf. *homosphaera*	Figure 7c	Spherical *Chlorella*-like cells with single chloroplasts without a pyrenoid. Cell diameter: 1.8-3.3 µm.
Mychonastes sp.	Figure 7b	For description see *Mychonastes* cf. *homosphaera* above, cell diameter: 1.8-3.3 µm.
Pseudomuriella cf. *schumacherensis*	Figure 7a	Spherical cells containing chloroplast (parietal saucer-shaped in young cells or divided into several segments in mature cells. Pyrenoids are absent. Cell diameter: 3.7-8.1 µm.
Scenedesmaceae sp.	Figure 6i	For description see *Desmodesmus* cf. *armatus* above, cell length: 5.3-8.4 µm.
Dilabifilum printzii	Figure 7i	For description see *Desmodesmus* cf. *armatus* above, cell length: 5.0-10.1 µm.
Hazenia mirabilis	Figure 7e	Individual cells contain single parietal chloroplast with a pyrenoid. Thalli heterotrichous, consisting of uniseriate, irregularly branched filaments. Cell length: 7.6-21.4 µm.
Pseudendocloniopsis botryoides	Figure 7f, 7g	Globose cells form roundish, irregular packets or heterotrichous thalli. Cells with single parietal chloroplast and a pyrenoid. Cell diameter: 4.0-11.1 µm.
Pseudendoclonium akinetum	Figure 7h	Globose cells forming uniseriate filaments, irregularly branched. Cells containing one parietal chloroplast with one or two pyrenoids. Cell length: 5.6-10.2 µm.

Table S3. Percentage identities (PID) to closest GenBank-relatives.

Identification of taxa based on 18S rDNA	Strain(s)	Closest relative(s) accession number(s)	Percentage identity	Habitat
Acutodesmus obliquus	D22-6-2B	FR865726	100	freshwater
Bracteacoccus	DB9-3	U63103	99.59	air-borne dust
Bracteacoccus aerius-relative	SAG 2403	U63101	98.59	air-borne dust
Chlamydomonas sp.	RK68	X53904	98.65	freshwater
Chlamydomonas sp.	RK68	JN903974	99.13	freshwater
Chlamydomonas sp.	RK68	AY781664	99.18	freshwater
Chlamydopodium vacuolatum	RK50+SAG 2402	M63001	98.77	n/a
Chlamydopodium vacuolatum	RK50+SAG 2402	GQ122367	99.82	freshwater
Chlorella sp.	RK52, D11-2	GQ487223	99.71	freshwater
Chlorella sp.	RK52/D11-2	FR865683, AY197628, FJ946888, DQ377324	99.90	freshwater
Chlorococcum cf. *sphacosum*	SAG 2398	JN968580/JN968584	99.94	freshwater
Chlorococcum ellipsoideum-relative1	GRK6-DB5	U70586	98.45	terrestrial (soil)
Chlorococcum ellipsoideum-relative2	SAG 2401/SAG 2400 /GRK6-DB6	U70586	98.33	terrestrial (soil)
Chlorococcum minutum-relative	SAG 2399	JN968585	97.65	terrestrial (soil)
Coccomyxa cf. *pringsheimii*	WB28	AY762603	99.61	terrestrial (phycobiont)
Cooccomyxa cf. *simplex*	WB40	FJ648514	99.83	freshwater
Desmochloris sp.	SAG 2397	FM882216/AB049416	99.30	freshwater
Desmodesmus cf. *armatus*	SAG 2395/RK43	FR865727	99.71	freshwater
Dilabifilum printzii	WB41 (PS)	KF144198	100	freshwater
Elliptochloris cf. *subsphaerica*	WB5-D1e	FJ648515, FJ648518	99.94	terrestrial (roof tile)
Hazenia sp.	SAG 2396	AF387156	99.94	freshwater
Marvania	WB67	AF124336	97.83	freshwater
Marvania	WB67	EF440182	99.30	terrestrial (permafrost)
Marvania	WB67	AY195983	99.60	freshwater
Monoraphidium terrestre cf. *dybowskii*	SAG 2393	Y16939	99.54	freshwater
Muriella terrestris	D6-DB2	AB012845	99.94	terrestrial (soil)
Muriella terrestris	D6-DB2	AY195969	100	freshwater
Mychonastes cf. *homosphaera*	RK48	GU799581	99.42	freshwater
Mychonastes sp.	DB6-29	AY543066	99.94	freshwater
Neocystis cf. *mucosa*	SAG 2405	HQ287929	99.56	terrestrial (soil)
Neocystis cf. *mucosa*	SAG 2405	HM565928	99.71	n/a
Pseudendocloniopsis botryoides	SAG 2394	AJ416102	99.76	terrestrial (soil)
Pseudendocloniopsis botryoides	SAG 2394	FR865755	99.94	freshwater
Pseudendoclonium akinetum	SAG 2404	AM087962	99.81	freshwater
Pseudendoclonium akinetum	SAG 2404	AM109906	99.75	freshwater
Pseudendoclonium akinetum	SAG 2404	DQ011230	99.94	freshwater
Pseudendoclonium basiliense	D9-14B	Z47996	99.82	freshwater
Pseudomuriella cf. *schumacherensis*	RK3	FR865690/X91268	99.01	freshwater/terrestrial (soil)
Pseudomuriella cf. *schumacherensis*	RK3	HQ292768	99.54	terrestrial (epiphytic)
Stichococcus bacillaris	WB74	AJ311637	99.88	freshwater
Stichococcus bacillaris	WB13	AJ311637	100	freshwater
Stichococcus cf. *deasonii*	WB38	DQ275460	99.74	terrestrial (soil)
Stichococcus mirabilis	WB69	AJ311638	100	n/a
Stichococcus sp.1	DB6-27	AB055866	99.71	n/a
Stichococcus sp.1	DB6-27, WB65	AB055866	100	n/a
Stichococcus sp.2	D4-2A	DQ275460	99.48	terrestrial (soil)
Stichococcus sp.3	SAG 2408	DQ275460	98.60	terrestrial (soil)
Stichococcus sp.4	SAG 2406	DQ275460	99.01	terrestrial (soil)

Legend. For each species, their closest available neighbouring sequences and pairwise sequence similarity (isolate/neighbouring sequence; in percentage calculated from p-distances) and the habitat from which the closest neighbours have been isolated are listed. The species are in two groups, i.e. one with high simililarities with neighbouring sequences (99.9 and 100.0 %) so that they represent the same species as the isolate, the other with lower sequence similarities so that the neighbouring sequences do not represent the same species as the isolate. "n.a." represents for "not applicable" where origin is not known.

Table S4. Sequences representing additional groups and lineages shown as collapsed triangles in **Figures 1-3**.

Figure	Taxon	GenBank accession number(s)
Figure 1	Chlorodendrophyceae	X68484, X70802
	Chlorophyceae	DQ078295, AY779858, M62861, M32703, COU83125
	Choricystis/Botryococcus	AY195970, AY762605, AY197640, AJ581912
	Desmococcus	AJ431571, EU434017
	Hegewaldia	FM205843, FM205844
	Lobosphaera clade	AB006051, EU878372, EU878374
	Oocystaceae	AF387154, AF228686, AF228691, AF228690, AF228689
	Parachlorella-clade	AB176664, AB037085, Y17470, X56105, FM205845
	Prasiola/Prasiolopsis/Trichophilus	AJ416106, EF200524, AY762600, AY762601
	Pseudochlorella/Koliella	AB006050, X63520, U18520, AF514410
	Pseudomarvania	AB087559, FJ896222
	Trebouxiales clade	Z28971, M62995, AB080310, Z21551
	Ulvophyceae	AB183610, AY278217
	Watanabea clade	AJ439401, X73991, FM946000, EU346910, X73998, EF595524
Figure 2	*Arenicolinia*	AB218701, AY271673, AF513371
	Chaetopeltidales	U83126, U83125, U83124
	Chaetophorales	AF182820, AF182816, AF182821, D86499
	Characiosiphonia	AF395437, AF395435
	Chlorodendrophyceae	X68484, X70802
	Chlorogonia	AJ410443, U70589
	Chloromonadinia	U57696, AJ410448, U57697
	Crucicarteria	D86501, U70595
	Dunaliellinia	EU589200, DQ009743
	Golenkinia	AF499924, AF499923
	Hafniomonas	AB248251, D86496
	Jenufa	HM563743, HM563744, AB257660, AB257659
	Moewusinia	U70782, AY220571
	Monadinia	AF514404, U57694
	Nephroselmidophyceae	X75565, X74754
	Oedogoniales	DQ018735, U83135, U83132
	Oogamochlamydinia	AJ410461, AJ410464, AJ410466, AB175836, AB175837
	Phacotinia	AY009898, AF395438
	Polytominia	U22941, AJ781310, U13984, U13985
	Radicarteria	AF182817, AF182819
	Sphaeropleales	see Fig.3 - Chlorophyceae which are not included in collapsed triangles
	Tatrensinia	AF514407, EF023280, AF514411
	Trebouxiophyceae	AY762602, AB017435
	Treubarinia	AY008844, U73471, U73474, AF277651
	Ulvophyceae	AY278217, AB183610
Figure 3	Chaetopeltidales	U83126, U83125, U83124
	Chaetophorales	AF182820, AF182816, AF182821, D86499
	Chlamyodomonadales	see Fig.2 - Chlorophyceae which are not included in collapsed triangles
	Chlorodendrophyceae	X68484, X70802
	Golenkinia	AF499924, AF499923
	Hydrodictyaceae	AY663044, AY663041, AY779858, M62997
	Jenufa	HM563743, HM563744, AB257660, AB257659
	Neochloridaceae	AF288361, AF288364, M62861, AJ581914, M63000
	Nephroselmidophyceae	X75565, X74754
	Oedogoniales	DQ018735, U83135, U83132
	Sphaeropleaceae	AF302770, AJU73469
	Trebouxiophyceae	AB017435, AY762602
	Ulvophyceae	AY278217, AB183610

Appendix | Chapter 3

Supporting Figures

Figure S1. ITS2 secondary structure analysis of *Chlorella vulgaris* and *C. pituita*.

Figure S2. Analysis of compensatory base changes (CBCs) in ITS2 sequences of *Chlorella*-like species.

Figure S3. 18S ML phylogeny of *Stichococcus*-like species.

Figure S4. ITS2 secondary structure analysis of *Stichococcus*-like species.

Figure S5. Neighbor-joining tree of *Stichococcus*-like species based on ITS2 sequences/secondary structures.

Figure S6. Microphotographs of representatives of the *Prasiola* clade.

Supporting Tables

Table S1. List of all analyzed accessions (isolates, strains and clones) of the polar *Chlorella*-like species and relatives.

Table S2. List of all analyzed accessions (isolates, strains and clones) of the *Stichococcus*-like species and relatives.

Table S3. List of the analyzed *Stichococcus*-like clades and their representative strains.

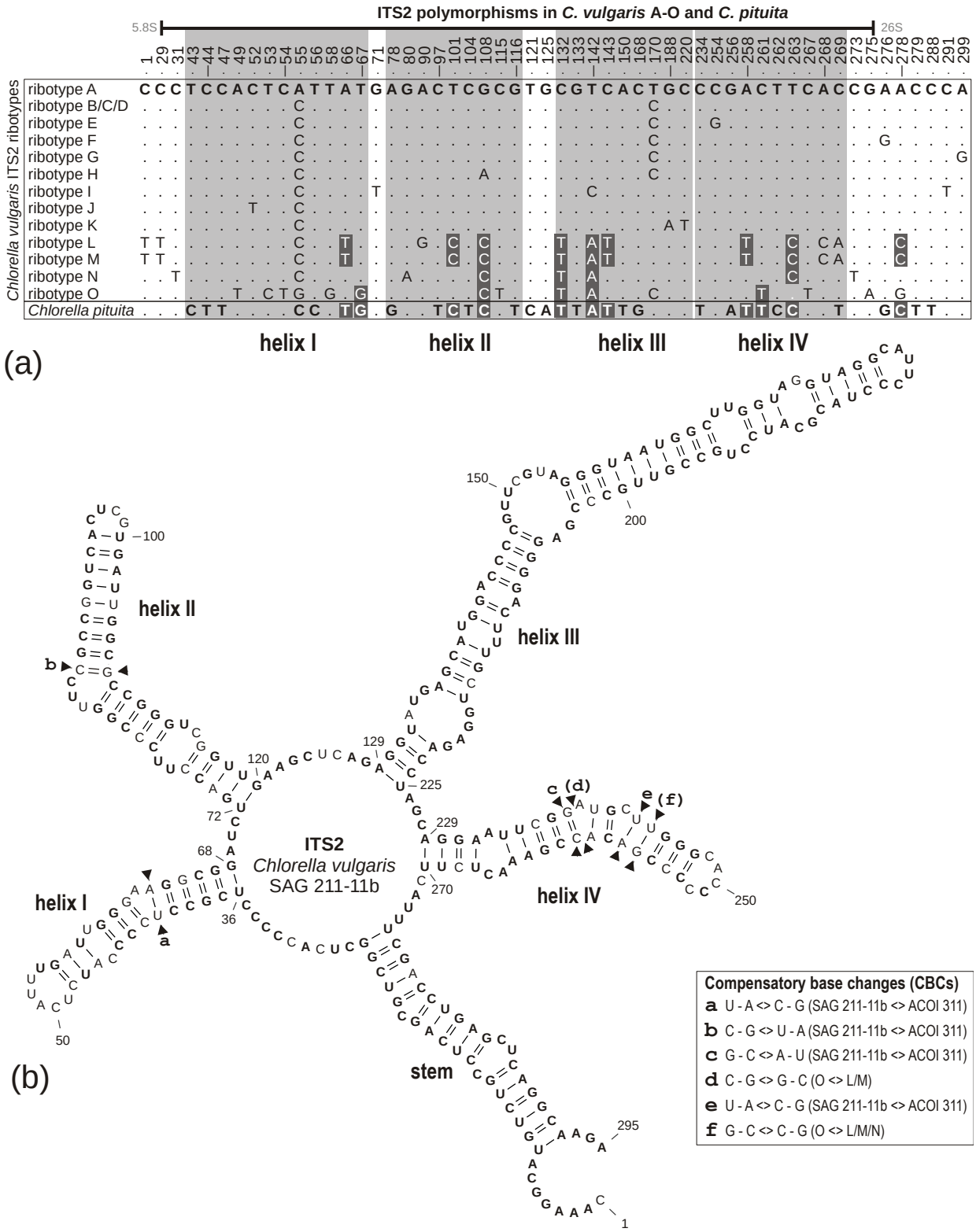


Figure S1. ITS2 secondary structure analysis of *Chlorella vulgaris* and *C. pituita*. (a) Polymorphic nucleotide sites detected in 13 ribotypes of *C. vulgaris* (ribotype A = SAG 211-11b) and in *C. pituita*. (b) ITS2 secondary structure model of *C. vulgaris* SAG 211-11b. Compensatory base changes (CBCs) between *C. vulgaris* and *C. pituita* (a, b, c, e) are marked by black triangles. Additional CBCs (d, f) among intraspecific ribotypes of *C. vulgaris* are shown as well. Bold letters represent nucleotide sites conserved in *C. vulgaris* and *C. pituita*.

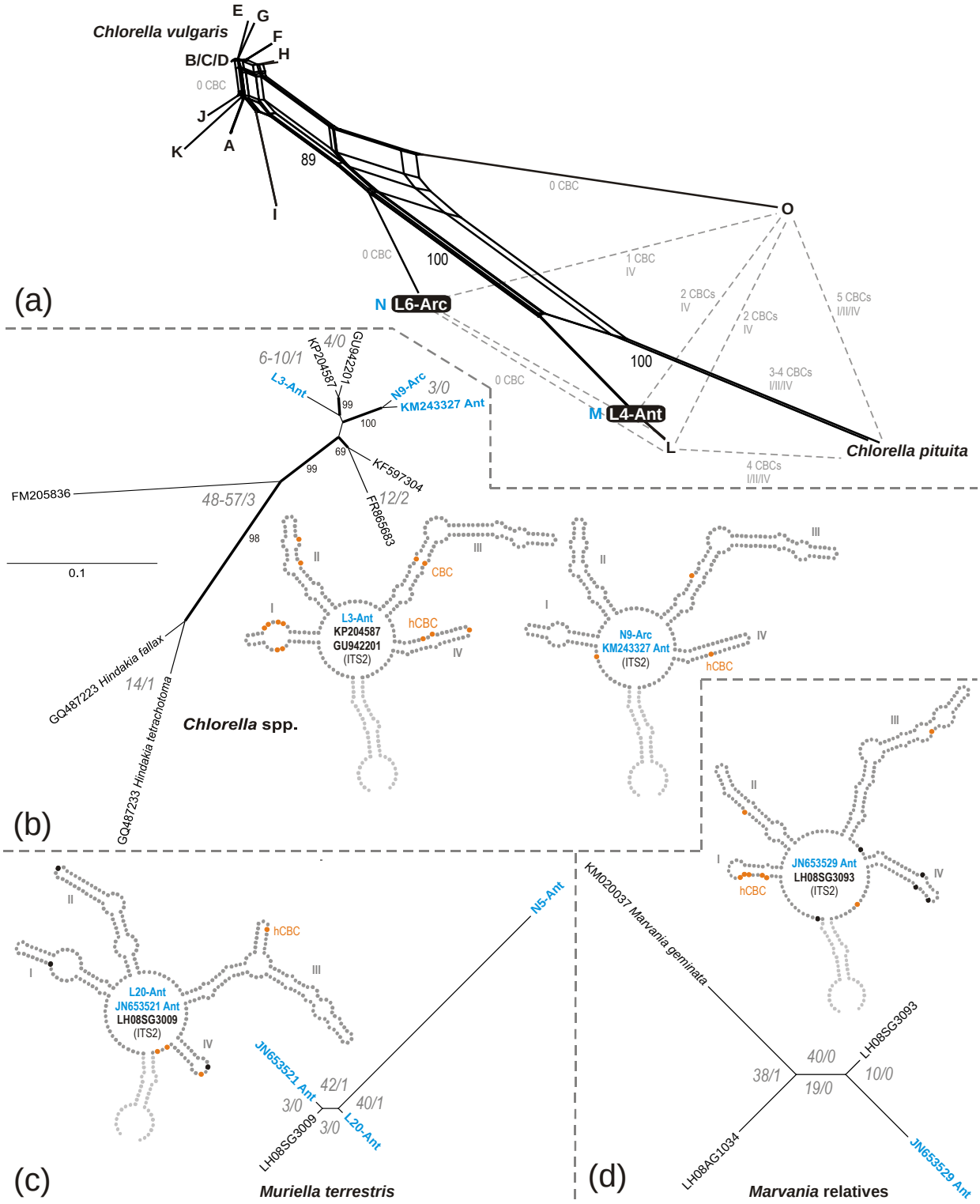


Figure S2. Analysis of compensatory and hemi-compensatory base changes (CBCs, hCBCs) in ITS2 sequences of *Chlorella*-like species. (a) Neighbor-net analysis of *Chlorella vulgaris* SAG 211-11b (ribotype A), other *C. vulgaris* ribotypes (B-O) and *C. pituita* ACOI 311 (**Fig. S1a**). The numbers close to the solid lines represent bootstrap support values; the numbers close to the dashed lines represent CBCs between two sequences (the respective helices (**Fig. S1b**) are given by Roman numerals). (b)-(d) Consensus secondary structures of closely related accessions of the Chlorellaceae: (b) *Chlorella* sp.1 and *C.* sp.2; neighbor-joining tree (based on ITS2 sequences and secondary structures) and consensus ITS2 secondary structure of accessions closely related to the polar strains. (c) *Muriella* terrestris. (d) *Marvania relatives*. Nucleotide positions (represented as dots) within the ITS2 secondary structures are colored as follows: grey dots=conserved positions, orange dots=nucleotide substitutions and black dots=nucleotide deletions. Grey numbers between sequences in the NJ-trees give nucleotide differences and compensatory base changes (CBCs), respectively.

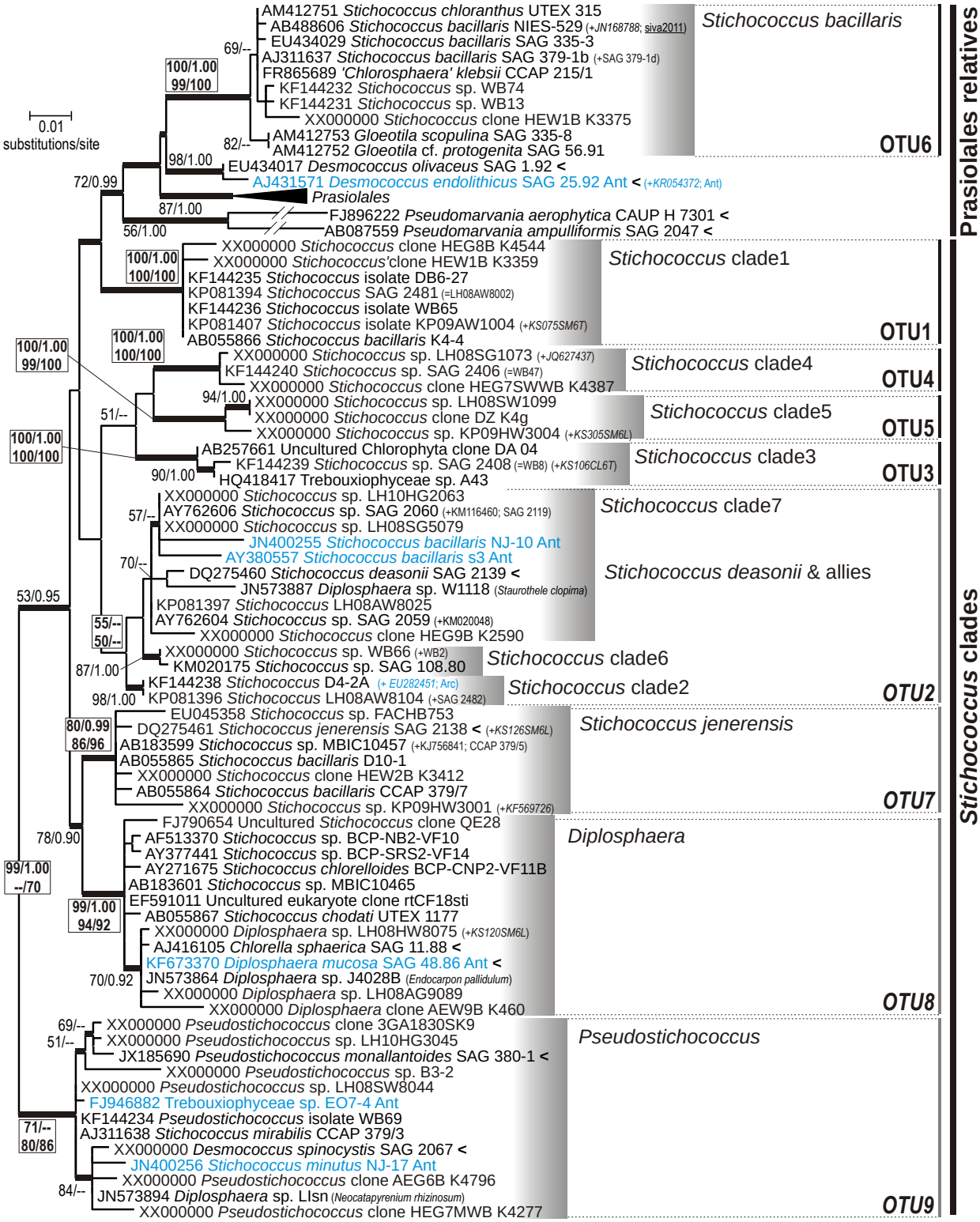


Figure S3. 18S ML phylogeny of *Stichococcus*-like species. Sequences of authentic strains are marked by a '<' sign. The numbers next to branches indicate statistical support values (maximum-likelihood bootstraps (ML)/Bayesian posterior probabilities (BI)); the clades of particular interest were additionally tested via maximum parsimony (MP) and bio-neighbor-joining (NJ) and the statistic support values are given in the following order: ML/BI/MP/NJ. Assignations into operational taxonomic units are based on sequence similarities ≥ 99.5% (= OTU) and ≥ 99.0% (= *OTU*).

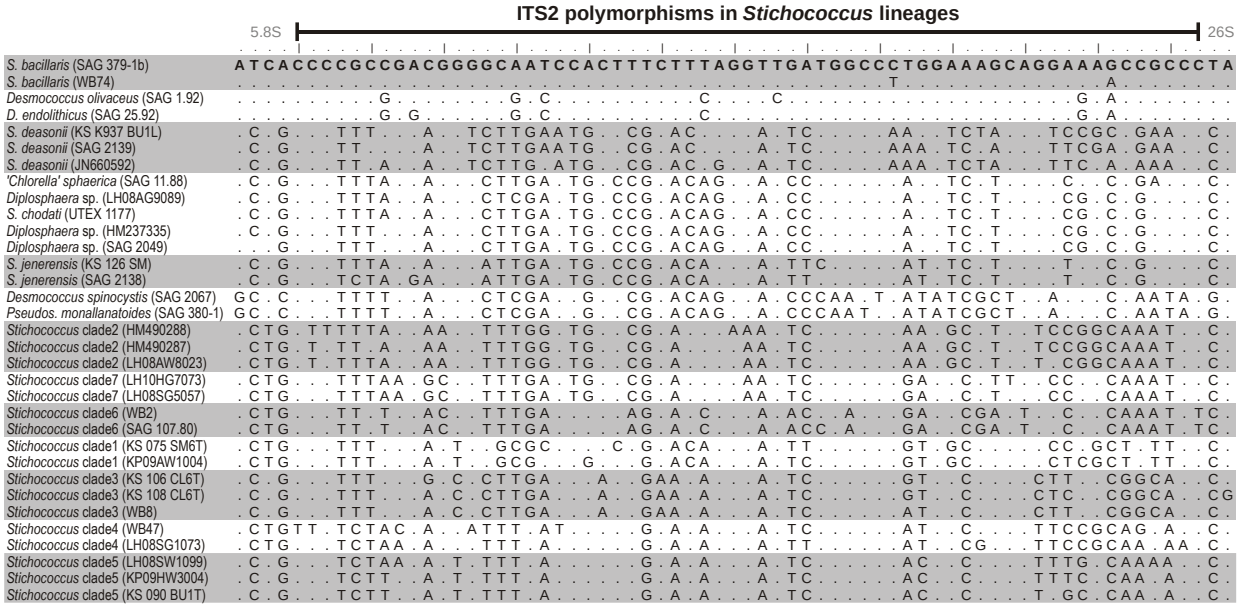


Figure S4. ITS2 secondary structure analysis of *Stichococcus*-like species. (a) Polymorphic nucleotide sites detected in 33 ribotypes of *Stichococcus*-like species and allies. (b) ITS2 secondary structure model of *S. bacillaris* SAG 379-1b. Bold letters represent nucleotide sites which are conserved across all 56 analyzed ITS2 accessions.

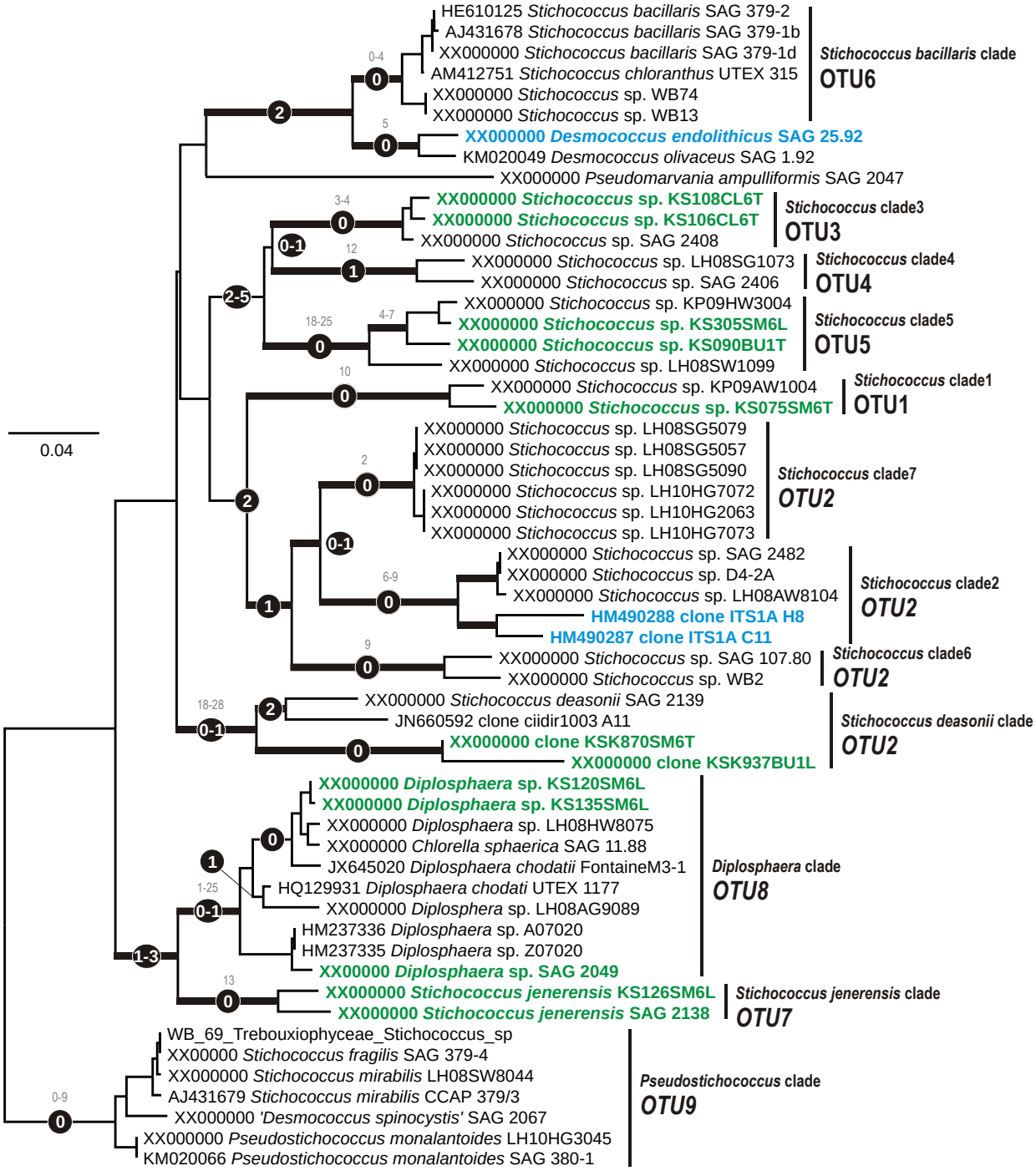


Figure S5. Neighbor-joining tree of *Stichococcus*-like species based on ITS2 sequences/secondary structures. Thick lines indicate bootstrap support values ≥ 80. Compensatory base changes (CBCs) detected within clades are given on branches as white numbers in black circles; the grey numbers above CBCs give nucleotide differences detected within clades. The polar and tropical accessions are blue and green colored, respectively.

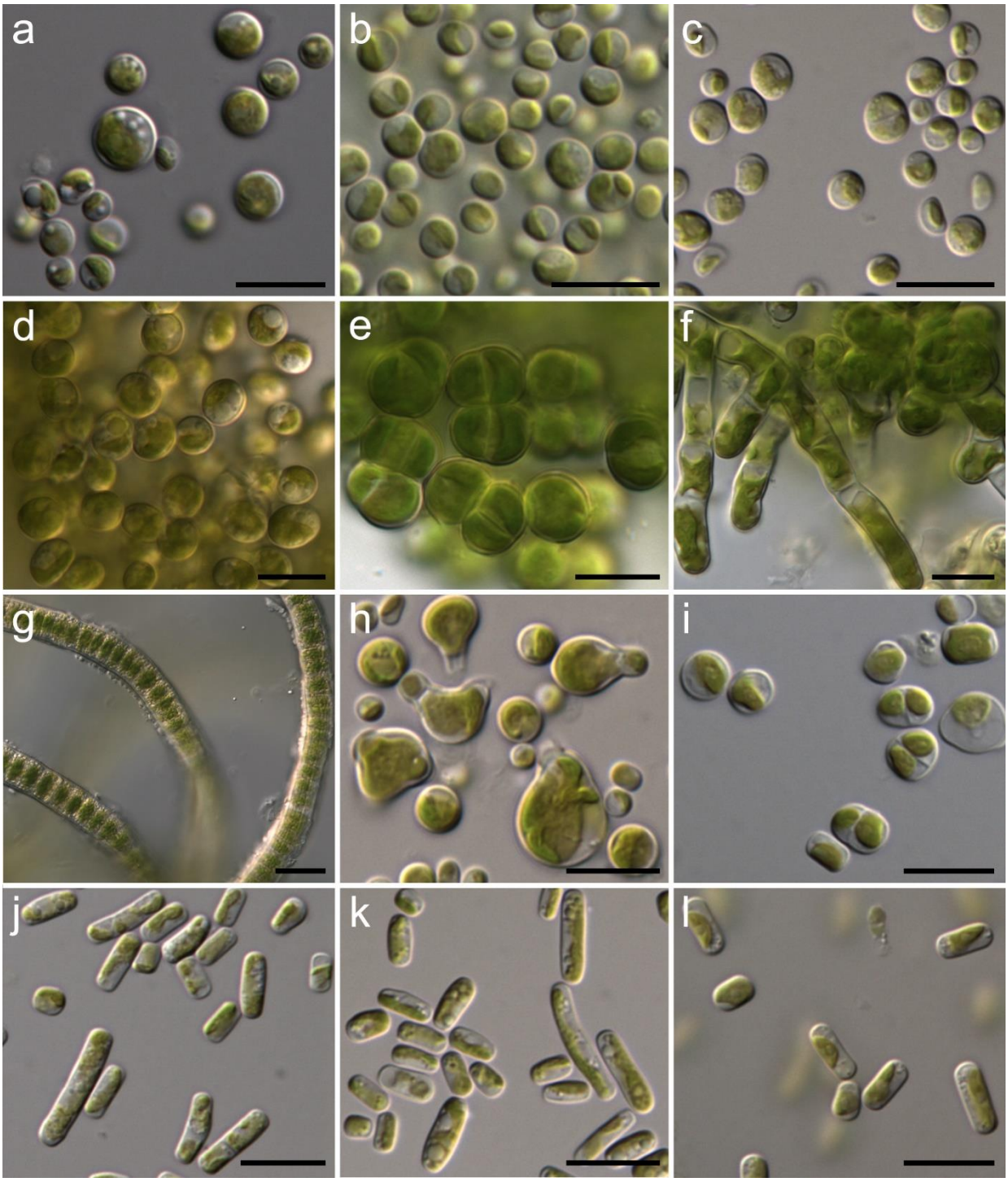


Figure S6. Microphotographs of representatives of the *Prasiola* clade. (a) *'Chlorella' mirabilis* SAG 38.88; (b) *'Chlorella' sphaerica* SAG 11.88; (c) *Diplosphaera* sp. SAG 2049; (d) *'Desmococcus' spinocystis* SAG 2067; (e) *Desmococcus olivaceus* SAG 1.92; (f) *Prasiolopsis ramosa* SAG 26.82; (g) *Prasola crispa* SAG 43.96; (h) *Pseudomarvania ampulliformis* SAG 2047; (i) *Stichococcus jenerensis* SAG 2138; (j) *Pseudostichococcus monallantoides* SAG 380-1; (k) *Stichococcus fragilis* SAG 379-4; (l) *Stichococcus deasonii* SAG 2139.

Table S1. List of all analyzed accessions (isolates, strains and clones) of the polar *Chlorella*-like species and relatives.

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Chlorella s.str.	*Chlorella pituita*	ACOI 311	GQ176852	GQ176853	-	PT (Mira)	freshwater (trout nursery)
Chlorella s.str.	*Chlorella pituita*	ACOI 856	FM205855	FM205856	-	PT (Manteigas)	freshwater
Chlorella s.str.	*Chlorella vulgaris*	3GSG1R K22	this study	n/a	N50°52'0.000" E10°50'0.000"	DE (Castle Gleichen)	subaerial (epilithic; sandstone)
Chlorella s.str.	*Chlorella vulgaris*	CCAP 211/79	FR865683	FR865683	-	UK (Edinburgh)	waste solvent biofilter
Chlorella s.str.	*Chlorella vulgaris*	DB1-10	KF144171	this study		DE (Deinschwanger Bach)	semi-terrestrial (creek biofilm)
Chlorella s.str.	*Chlorella vulgaris*	HEG7SWWB K4396	this study	n/a	N51°16'24.897" E10°24'37.485"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris*	L1	this study	n/a	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Chlorella s.str.	*Chlorella vulgaris*	L5	this study	n/a	N79°08'0.000" W80°30'0.000"	CA (Ellesmere Island)	soil (close to river)
Chlorella s.str.	*Chlorella vulgaris*	NJ-7	DQ377323	n/a	-	AQ (Zhongshan Station)	terrestrial (wet rocks)
Chlorella s.str.	*Chlorella vulgaris* A	CCAP 211/11B	n/a	AY591507	-	NL (Delft)	freshwater
Chlorella s.str.	*Chlorella vulgaris* A	CCAP 254/5	FR865696	FR865697	-	US (Bloomington, Indiana)	freshwater (aquarium)
Chlorella s.str.	*Chlorella vulgaris* A	SAG 211-11b	FM205832	FM205832	-	NL (Delft)	freshwater
Chlorella s.str.	*Chlorella vulgaris* B	CCAP 211/110	FN298918	FN298918	-	US (n/a)	freshwater (*Paramecium* symbiont)
Chlorella s.str.	*Chlorella vulgaris* B	CCAP 211/74	FR865682	FR865682	-	UK (Cumbria)	freshwater (Esthwaite Water)
Chlorella s.str.	*Chlorella vulgaris* B	CCAP 211/80	FM205853	FM205853	-	DE (Elsnigk)	freshwater (Molkerteich)
Chlorella s.str.	*Chlorella vulgaris* B	CCAP 211/81	FM205854	FM205854	-	DE (Trebbichau)	freshwater (Salzteich)
Chlorella s.str.	*Chlorella vulgaris* B	LH08HG4032	this study	this study	N51°6'48.104" E10°26'10.249"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH08HG4088	this study	this study	N51°6'48.104" E10°26'10.249"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH08HG5074	this study	this study	N51°12'57.220" E10°19'21.096"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH08HW9094	this study	this study	N51°7'48.871" E10°22'52.139"	DE (Hainich-Dün)	soil (forest)
Chlorella s.str.	*Chlorella vulgaris* B	LH08SG1071	this study	this study	N53°5'14.712" E13°58'10.717"	DE (Schorfheide-Chorin)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH08SG3006	this study	this study	N53°6'10.204" E13°59'8.519"	DE (Schorfheide-Chorin)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH10HG2049	this study	this study	N51°0'2.696" E10°25'48.036"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH10HG6014	this study	this study	N51°12'53.766" E10°23'28.395"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH10HG6019	this study	this study	N51°12'53.766" E10°23'28.395"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	LH10HG9075	this study	this study	N51°13'26.031" E10°22'50.834"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* B	NB-1	n/a	KC840685	-	CN?	n/a
Chlorella s.str.	*Chlorella vulgaris* E	SAG 9.88	n/a	AY591500	-	ES (Madrid)	freshwater (waste-water)
Chlorella s.str.	*Chlorella vulgaris* F	CCAP 211/11P	FR865658	FR865658	-	SE (Lund)	freshwater (pond in park)
Chlorella s.str.	*Chlorella vulgaris* F	SAG 211-11p	n/a	AY591505	-	SE (Lund)	freshwater (pond in park)
Chlorella s.str.	*Chlorella vulgaris* G	TW-1	n/a	KC840684	-	CN(?)	n/a
Chlorella s.str.	*Chlorella vulgaris* H	CCAP 211/82	FM205855	FM205855	-	DE (Micheln)	freshwater
Chlorella s.str.	*Chlorella vulgaris* H	LH08HG5082	this study	this study	N51°12'57.220" E10°19'21.096"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* H	LH10HG6052	this study	this study	N51°12'53.766" E10°23'28.395"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* I	CCAP 211/63	FR865681	FR865681	-	UK (Cambridge)	freshwater (Peterhouse Ditch)
Chlorella s.str.	*Chlorella vulgaris* I	LH08HG1081	this study	this study	N50°58'17.934" E10°24'19.306"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* J	CCAP 211/109	FN298917	FN298917	-	US	freshwater (*Paramecium* symbiont)

Table S1. (continuation)

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Chlorella s.str.	*Chlorella vulgaris* K	D2	JX185297	JX185298	-	CN(?)	n/a
Chlorella s.str.	*Chlorella vulgaris* L	LH10HG2067	this study	this study	N51°0'2.696" E10°25'48.036"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* L	LH10HG2081	this study	this study	N51°0'2.696" E10°25'48.036"	DE (Hainich-Dün)	soil (grassland)
Chlorella s.str.	*Chlorella vulgaris* M	L4	this study	this study	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Chlorella s.str.	*Chlorella vulgaris* N	L6	this study	this study	N79°08'0.000" W80°30'0.000"	CA (Ellesmere Island)	soil (moraine)
Chlorella s.str.	*Chlorella vulgaris* O	LH10HG1069	this study	this study	N50°58'17.934" E10°24'19.306"	DE (Hainich-Dün)	soil (grassland)
Chlorella sp.	*Chlorella* sp.	KNUA034	KM243327	KM243327	-	AQ	freshwater
Chlorella sp.	*Chlorella* sp.2	L3	this study	this study	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Chlorella sp.	*Chlorella* sp.1	N9	this study	this study	N79°58'0.000" E11°21'0.000"	NO (Svalbard)	soil (deglaciated)
Chlorella sp.	*Chlorella* sp.	NDem 9/21	AY197628	n/a	-	US	freshwater
Chlorella sp.	*Chlorella* sp.	NJ-18	DQ377324	n/a	-	AQ (Zhongshan Station)	terrestrial (wet rocks)
Chlorella sp.	*Chlorella* sp.	NMX37N	JF767012	n/a	N41.48 E112.63	CN?	freshwater
Chlorella sp.	*Chlorella* sp.	RK52	KF144182	n/a	-	DE (Deinschwanger Bach)	freshwater biofilm
Chlorella sp.	*Chlorella* sp.	VPL9-6	FJ946888	n/a	-	AQ (Trinity Peninsula)	freshwater
Chlorella sp.	Chlorellaceae	MCWWW9	KP204587	KP204587	-	CA (Nova Scotia)	freshwater (wastewater)
Chlorella sp.	Chlorellaceae	N505T04	GU942201	GU942201	-	CN (South China Sea)	marine
Chlorella sp.	*Micractinium inerimum*	NLP-F014	KF597304	KF597304	-	KR	freshwater (wastewater)
Chlorellaceae	Chlorellaceae	L24	this study	n/a	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Hindakia	*Hindakia fallax*	CCAP 222/29	GQ487223	GQ487223	-	-	-
Hindakia	*Hindakia tetrachotoma*	CCAP 222/80	GQ487233	GQ487233	-	-	-
Micractinium	*Micractinium pusillum*	CCAP 248/5	FM205836	FM205836	-	-	-
Muriella	Chlorophyta	clone WIM107	AM114820	n/a	-	-	-
Muriella	*Muriella* sp.	AS 2-4	AY195969	n/a	-	-	-
Muriella	*Muriella terrestris*	AEG6B K4797	this study	n/a	N48°23'52.818" E9°20'31.152"	DE (Schwäbische Alb)	soil (grassland)
Muriella	*Muriella terrestris*	ASIB V38	AB012845	n/a	-	-	-
Muriella	*Muriella terrestris*	clone Ant 8/104	n/a	JN653521	-	AQ	soil (permafrost)
Muriella	*Muriella terrestris*	D6-DB2	KF144209	n/a	-	-	-
Muriella	*Muriella terrestris*	L20	this study	this study	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Muriella	*Muriella terrestris*	LH08SG3009	this study	this study	N53°6'10.204" E13°59'8.519"	DE (Schorfheide-Chorin)	soil (grassland)
Muriella	*Muriella terrestris*	LH10HG9077	this study	n/a	N51°13'26.031" E10°22'50.834"	DE (Hainich-Dün)	soil (grassland)
Muriella	*Muriella terrestris*	N5	this study	this study	S67°34'0.000" W68°08'0.000"	AQ (Adelaide Island)	soil (rookeries)
Nannochloris-like	*Marvania geminata*	SAG 12.88	AF124336	KM020037	-	-	-
Nannochloris-like	*Chlorella* sp.	193-GA188	EU282456	n/a	-	RU (Siberia, Kolyma)	soil (permafrost)
Nannochloris-like	*Marvania* relative1	clone Ant 8/117	n/a	JN653529	-	AQ	soil (permafrost)
Nannochloris-like	*Marvania* relative	HEG9 B K25502	this study	n/a	N51°13'26.031" E10°22'50.834"	DE (Hainich-Dün)	soil (grassland)
Nannochloris-like	*Marvania* relative1	L13	this study	n/a	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Nannochloris-like	*Marvania* relative1	L14	this study	n/a	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Nannochloris-like	*Marvania* relative1	L15	this study	n/a	S67°36'0.000" W68°15'0.000"	AQ (Anchorage Island)	soil (bare ground)
Nannochloris-like	*Marvania* relative1	L23	this study	n/a	S67°32'0.000" W68°07'0.000"	AQ (Killingbeck Island)	endozoic (gut content; *Cryptopygus*)
Nannochloris-like	*Marvania* relative1	L32	this study	n/a	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)

Table S1. (continuation)

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Nannochloris-like	*Marvania* relative2	LH08AG1034	this study	this study	N48°23'52.818" E9°20'31.152"	DE (Schwäbische Alb)	soil (grassland)
Nannochloris-like	*Marvania* relative1	LH08SG2053	this study	n/a	N53°5'21.505" E13°58'48.169"	DE (Schorfheide-Chorin)	soil (grassland)
Nannochloris-like	*Marvania* relative1	LH08SG3078	this study	this study	N53°6'10.204" E13°59'8.519"	DE (Schorfheide-Chorin)	soil (grassland)
Nannochloris-like	*Marvania* relative1	LH08SG3093	this study	this study	N53°6'10.204" E13°59'8.519"	DE (Schorfheide-Chorin)	soil (grassland)
Nannochloris-like	*Marvania* relative1	LH10HG2094	this study	n/a	N51°0'2.696" E10°25'48.036"	DE (Hainich-Dün)	soil (grassland)
Nannochloris-like	*Marvania* sp.	WB67	KF144207	n/a	-	-	-
Nannochloris-like	*Nannochloris coccoides*	CCAP 251/1b	AB080301	n/a	-	-	-
Nannochloris-like	*Nannochloris* sp.	An-1	EF440182	n/a	-	-	-
Nannochloris-like	*Nannochloris* sp.	JL 4-6	AY195983	n/a	-	-	-

Legend. AQ=Antarctica, CA=Canada, CN=China, DE=Germany, ES=Spain, KR=South Korea, NL=Netherlands, NO=Norway, PT=Portugal, RU=Russia, SE=Sweden, UK=United Kingdom, US=United States of America.

Table S2. List of all analyzed accessions (isolates, strains and clones) of the *Stichococcus*-like species and relatives.

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Chlorella mirabilis	*Chlorella mirabilis*	KFFB12-1	this study	this study	S4°6'49.852" W78°58'1.012"	EC (Bombuscaro)	subaerial (litter)
Chlorella mirabilis	*Chlorella mirabilis*	L10	this study	n/a	S62°10'0.000" W58°30'0.000"	AQ (King George Island)	soil (deglaciated)
Chlorella mirabilis	*Chlorella mirabilis*	LH10HG6139	this study	n/a	N51°12'53.766" E10°23'28.395"	DE (Hainich-Dün)	soil (grassland)
Chlorella mirabilis	*Chlorella mirabilis*	Andreyeva 748-I	X74000	n/a	-	RU	tundra
Chlorella mirabilis	*Chlorella mirabilis*	LH8AG9040	this study	this study	N48°23'40.815" E9°30'10.053"	DE (Schwäbische Alb)	soil (grassland)
Chlorella mirabilis	*Chlorella mirabilis*	SEG5B K5318	this study	n/a	N53°6'26.830" E14°0'1.885"	DE (Schorfheide-Chorin)	soil (grassland)
Desmococcus	*Desmococcus endolithicus*	clone EN5JG	KR054372	n/a	-	-	-
Desmococcus	*Desmococcus endolithicus*	SAG 25.92	EU434026	this study	-	AQ (Marie Bird Land)	subaerial (chasmoendolithic)
Desmococcus	*Desmococcus olivaceus*	SAG 1.92	EU434017	KM020049		AT (Vienna)	subaerial (epixylic; tree bark)
Diplosphaera	*Stichococcus* sp.	BCP-NB2-VF10	AF513370	n/a	-	-	-
Diplosphaera	*Stichococcus* sp.	BCP-SRS2-VF14	AY377441	n/a	-	-	-
Diplosphaera	*Diplosphaera*	clone rtCF18sti	EF591011	n/a	-	-	freshwater
Diplosphaera	*Diplosphaera*	MBIC10465	AB183601	n/a	-	-	-
Diplosphaera	*Diplosphaera chodatii*	FontaineM3-1	n/a	JX645020	-	AT (Waldaist)	subaerial (*Dermatocarpon* symboint)
Diplosphaera	*Diplosphaera* sp.	A07020	n/a	HM237336	-	CN (Shapotou desert)	subaerial (*Endocarpon* symbiont)
Diplosphaera	*Diplosphaera* sp.	AEW9B K460	this study	n/a	N48°22'09.600" E9°24'54.800"	DE (Schwäbische Alb)	soil (forest)
Diplosphaera	*Diplosphaera* sp.	Z07020	n/a	HM237335	-	CN (Shapotou desert)	subaerial (*Endocarpon* symbiont)
Diplosphaera	*Stichococcus chlorelloides*	BCP-CNP2-VF11B	AY271675	n/a	-	-	-
Diplosphaera	*Stichococcus* sp.	clone QE28	FJ790654	n/a	-	-	-
Diplosphaera	*Chlorella sphaerica*	SAG 11.88	AJ416105	this study	-	NZ (Waweira Scenic Reserve)	subaerial (phycobiont)
Diplosphaera	*Stichococcus chodati*	UTEX 1177	AB055867	n/a	-	-	-
Diplosphaera	*Diplosphaera chodati*	UTEX 1177	AB055867	HQ129931	-	US (Texas)	soil (blackland prairie soil)
Diplosphaera	*Diplosphaera mucosa*	SAG 48.86	KF673370	n/a	-	AQ (Princess Elizabeth Land)	subaerial (moss)
Diplosphaera	*Diplosphaera* sp.	J4028B	JN573864	n/a	-	-	-
Diplosphaera	*Diplosphaera* sp.	KS120SM6L	this study	this study	S3°58'31.436" W79°4'17.341"	EC (San Francisco)	subaerial (epiphytic; leaves)
Diplosphaera	*Diplosphaera* sp.	KS135SM6L	n/a	this study	S3°58'31.436" W79°4'17.341"	EC (San Francisco)	subaerial (epiphytic; leaves)

Table S2. (continuation)

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Diplosphaera	*Diplosphaera* sp.	LH08AG9089	this study	this study	N48°23'40.815" E9°30'10.053"	DE (Schwäbische Alb)	soil (grassland)
Diplosphaera	*Diplosphaera* sp.	LH08HW8075	this study	this study	N51°21'20.852" E10°31'1.083"	DE (Hainich-Dün)	soil (forest)
Diplosphaera	*Diplosphaera* sp.	SAG 2049	n/a	this study	S8°16'25.200" E115°09'57.900"	ID (Bali, Lake Bratan)	freshwater
Pseudomarvania	*Pseudomarvania aerophytica*	CAUP H 7301	FJ896222	n/a	-	-	-
Pseudomarvania	*Pseudomarvania ampulliformis*	SAG 2047	AB087559	this study	-	JP (Taishaku-kyo Gorge)	subaerial (epixylic; tree bark)
Pseudostichococcus	*Pseudostichococcus* sp.	LH08SW8044	this study	this study	N53°11'30.470" E13°55'49.216"	DE (Schorfheide-Chorin)	soil (forest)
Pseudostichococcus	*Pseudostichococcus* sp.	WB69	KF144234	this study	-	DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Pseudostichococcus	*Stichococcus mirabilis*	CCAP 379/3	AJ311638	AJ431679	-	n/a	n/a
Pseudostichococcus	Trebouxiophyceae sp.	EO7-4	FJ946882	n/a	-	AQ (East Ongul)	freshwater
Pseudostichococcus	*Pseudostichococcus monalantoides*	SAG 380-1	JX185690	KM020066	n/a	DE	freshwater (culture; *Enteromorpha*)
Pseudostichococcus	*Pseudostichococcus* sp.	3GA1830SK9	this study	n/a	N50°52'0.000" E10°50'0.000"	DE (Castle Gleichen)	subaerial (epilithic; sandstone)
Pseudostichococcus	*Pseudostichococcus* sp.	B3-2	this study	n/a	N54°11'0.000" E7°53'0.000"	DE (Helgoland)	subaerial (epilithic; bunker wall)
Pseudostichococcus	*Pseudostichococcus* sp.	LH10HG3045	this study	this study	N50°59'53.129" E10°25'58.616"	DE (Hainich-Dün)	soil (grassland)
Pseudostichococcus	*Stichococcus fragilis*	SAG 379-4	n/a	this study	N41°35'58.300" W70°34'51.900"	US (Massachusetts)	freshwater (aquarium)
Pseudostichococcus	*Desmococcus spinocystis*	SAG 2067	this study	this study	N43°45'07.700" E15°22'10.300"	HR (Island of Lavsa)	soil
Pseudostichococcus	*Diplosphaera* sp.	Lsn	JN573894	n/a	-	-	-
Pseudostichococcus	*Pseudostichococcus* sp.	AEG6B K4796	this study	n/a	N48°24'4.600" E9°26'30.100"	DE (Schwäbische Alb)	soil (grassland)
Pseudostichococcus	*Pseudostichococcus* sp.	HEG7MW B K4277	this study	n/a	N51°16'24.897" E10°24'37.485"	DE (Hainich-Dün)	soil (grassland)
Pseudostichococcus	*Stichococcus minutus*	NJ-17	JN400256	n/a	-	AQ (Zhongshan Station)	terrestrial (wet rocks)
Stichococcus bacillaris	*Chlorosphaera klebsii*	CCAP 215/1	FR865689	FR865689	-	-	-
Stichococcus bacillaris	*Gloeotila* cf. *protogenita*	SAG 56.91	AM412752	AM412752	-	-	-
Stichococcus bacillaris	*Gloeotila scopulina*	SAG 335-8	AM412753	AM412753	-	-	-
Stichococcus bacillaris	*Stichococcus bacillaris*	HEW1B K3375	this study	n/a	N51°11'7.278" E10°19'25.036"	DE (Hainich-Dün)	soil (forest)
Stichococcus bacillaris	*Stichococcus bacillaris*	NIES-529	AB488606	n/a	-	-	-
Stichococcus bacillaris	*Stichococcus bacillaris*	RCC 1054	KF899844	n/a	-	-	-
Stichococcus bacillaris	*Stichococcus bacillaris*	SAG 335-3	EU434029	n/a	-	-	-
Stichococcus bacillaris	*Stichococcus bacillaris*	SAG 379-1b	AJ311637	AJ431678	-	CH (Basel)	freshwater (water dish)

Table S2. (continuation)

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Stichococcus bacillaris	*Stichococcus bacillaris*	SAG 379-1d	n/a	this study	n/a	US (Alaska)	n/a
Stichococcus bacillaris	*Stichococcus bacillaris*	SAG 379-2	HE610125	HE610125	-	DE (Bernburg)	n/a
Stichococcus bacillaris	*Stichococcus bacillaris*	siva2011	JN168788	n/a	-	-	-
Stichococcus bacillaris	*Stichococcus bacillaris*	UTEX 315	AM412751	AM412751	-	DE (Bernburg)	n/a
Stichococcus bacillaris	*Stichococcus bacillaris*	WB13	KF144231	this study		DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Stichococcus bacillaris	*Stichococcus bacillaris*	WB74	KF144232	this study		DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Stichococcus clade1	*Stichococcus bacillaris*	K4-4	AB055866	n/a	-	-	-
Stichococcus clade1	*Stichococcus* sp.	DB6-27	KF144235	n/a	-	-	-
Stichococcus clade1	*Stichococcus* sp.	HEG8B K4544	this study	n/a	N51°16'16.527" E10°25'4.6"	DE (Hainich-Dün)	soil (grassland)
Stichococcus clade1	*Stichococcus* sp.	HEW1B K3359	this study	n/a	N51°11'7.278" E10°19'25.036"	DE (Hainich-Dün)	soil (forest)
Stichococcus clade1	*Stichococcus* sp.	KP09AW1004	KP081407	this study		DE (Schwäbische Alb)	subaerial (epixylic; tree bark)
Stichococcus clade1	*Stichococcus* sp.	KS075SM6T	this study	this study	S3°58'31.436" W79°4'17.341"	EC (San Francisco)	subaerial (epixylic; tree bark)
Stichococcus clade1	*Stichococcus* sp.	SAG 2481 (=LH08AW8002)	KP081394	n/a	-	DE (Schwäbische Alb)	soil (forest)
Stichococcus clade1	*Stichococcus* sp.	WB65	KF144236	n/a	-	-	-
Stichococcus clade2	*Stichococcus* sp.	594-GA18	EU282451	n/a	-	RU (Siberia, Kolyma)	soil (permafrost)
Stichococcus clade2	*Stichococcus* sp.	D4-2A	KF144238	this study	-	DE (Deinschwanger Bach)	semi-terrestrial (creek biofilm)
Stichococcus clade2	*Stichococcus* sp.	ITS1A C11	n/a	HM490287	-	AQ (Miers Valley)	subaerial (hypolithic)
Stichococcus clade2	*Stichococcus* sp.	ITS1A H8	n/a	HM490288	-	AQ (Miers Valley)	subaerial (hypolithic)
Stichococcus clade2	*Stichococcus* sp.	LH08AW8104	KP081396	this study	-	DE (Schwäbische Alb)	soil (forest)
Stichococcus clade2	*Stichococcus* sp.	SAG 2482 (=LH08AW8023)	KP081395	this study	-	DE (Schwäbische Alb)	soil (forest)
Stichococcus clade3	*Stichococcus* sp.	A43	HQ418417	n/a	-	-	soil; Yellowstone NP
Stichococcus clade3	*Stichococcus* sp.	clone: DA-04	AB257661	n/a	-	-	rock surface, Swiss Alps
Stichococcus clade3	*Stichococcus* sp.	KS106CL6T	this study	this study	S4°6'52.681" W79°10'30.940"	EC (Cajanuma)	subaerial (epixylic; tree bark)
Stichococcus clade3	*Stichococcus* sp.	KS108CL6T	n/a	this study	S4°6'52.681" W79°10'30.940"	EC (Cajanuma)	subaerial (epixylic; tree bark)
Stichococcus clade3	*Stichococcus* sp.	SAG 2408 (=WB8)	KF144239	this study	-	DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Stichococcus clade4	*Stichococcus* sp.	clone B1_3_1E_88	JQ627437	n/a	-	-	-
Stichococcus clade4	*Stichococcus* sp.	HEG7SWWBK4387	this study	n/a	N51°16'24.897" E10°24'37.485"	DE (Hainich-Dün)	soil (grassland)
Stichococcus clade4	*Stichococcus* sp.	LH08SG1073	this study	this study	N53°5'14.712" E13°58'10.717"	DE (Schorfheide-Chorin)	soil (grassland)
Stichococcus clade4	*Stichococcus* sp.	SAG 2406 (=WB47)	KF144240	this study	-	DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Stichococcus clade5	*Stichococcus* sp.	DZ K4g	this study	n/a	N51°33'29.700" E9°56'20.300"	DE (Göttingen)	subaerial (epilithic; roof tile)

Table S2. (continuation)

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Stichococcus clade5	*Stichococcus* sp.	KP09HW3004	this study	this study	N51°16'17.900" E10°18'38.700"	DE (Hainich-Dün)	subaerial (epixylic; tree bark)
Stichococcus clade5	*Stichococcus* sp.	KS090BU1T	n/a	this study	S4°6'49.852" W78°58'1.012"	EC (Bombuscaro)	subaerial (epixylic; tree bark)
Stichococcus clade5	*Stichococcus* sp.	KS305SM6L	this study	this study	S3°58'31.436" W79°4'17.341"	EC (San Francisco)	subaerial (epiphytic; leaves)
Stichococcus clade5	*Stichococcus* sp.	LH08SW1099	this study	this study	N52°54'3.050" E13°50'46.921"	DE (Schorfheide-Chorin)	soil (forest)
Stichococcus clade6	*Stichococcus* sp.	SAG 107.80	n/a	this study	N45°15'33.900" W62°56'0.100"	CA (Nova Scotia)	n/a
Stichococcus clade6	*Stichococcus* sp.	SAG 108.80	KM020175	n/a	-	-	-
Stichococcus clade6	*Stichococcus* sp.	WB2	n/a	this study	N51°45'0.000" E10°5'0.000"	DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Stichococcus clade6	*Stichococcus* sp.	WB66	this study	n/a	N51°45'0.000" E10°5'0.000"	DE (Westerhöfer Bach)	semi-terrestrial (creek biofilm)
Stichococcus clade7	*Stichococcus* sp.	W1118	JN573887	n/a	-	-	-
Stichococcus clade7	*Stichococcus bacillaris*	NJ-10	JN400255	n/a	-	AQ (Zhongshan Station)	terrestrial (wet rocks)
Stichococcus clade7	*Stichococcus bacillaris*	s3	AY380557	n/a	-	AQ	soil
Stichococcus clade7	*Stichococcus* sp.	FG2/4.2	KM020048	KM020048	-	-	-
Stichococcus clade7	*Stichococcus* sp.	HEG9B K2590	this study	n/a	N51°13'26.031" E10°22'50.834"	DE (Hainich-Dün)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	LH08AW8025	KP081397	n/a	-	DE (Schwäbische Alb)	soil (forest)
Stichococcus clade7	*Stichococcus* sp.	LH08SG5057	this study	this study	N53°6'26.830" E14°0'1.885"	DE (Schorfheide-Chorin)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	LH08SG5079	this study	this study	N53°6'26.830" E14°0'1.885"	DE (Schorfheide-Chorin)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	LH08SG5090	this study	this study	N53°6'26.830" E14°0'1.885"	DE (Schorfheide-Chorin)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	LH10HG2063	this study	this study	N51°0'2.696" E10°25'48.036"	DE (Hainich-Dün)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	LH10HG7072	this study	this study	N51°16'24.897" E10°24'37.485"	DE (Hainich-Dün)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	LH10HG7073	this study	this study	N51°16'24.897" E10°24'37.485"	DE (Hainich-Dün)	soil (grassland)
Stichococcus clade7	*Stichococcus* sp.	SAG 2059	AY762604	n/a	-	-	-
Stichococcus clade7	*Stichococcus* sp.	SAG 2060	AY762606	n/a	-	-	-
Stichococcus clade7	*Stichococcus* sp.	SAG 2119	KM116460	n/a	-	-	-
Stichococcus deasonii	*Stichococcus deasonii*	SAG 2139	DQ275460	this study	-	US (Dauphin Island)	soil
Stichococcus deasonii	*Stichococcus* sp.	ciidir1003 A11	n/a	JN660592	-	MX	soil (rhizosphere from commercial field)
Stichococcus deasonii	*Stichococcus* sp.	KSK870SM6T	n/a	this study	S3°58'31.436" W79°4'17.341"	EC (San Francisco)	subaerial (epixylic; tree bark)
Stichococcus deasonii	*Stichococcus* sp.	KSK937BU1L	n/a	this study	S4°6'49.852" W78°58'1.012"	EC (Bombuscaro)	subaerial (epiphytic; leaves)
Stichococcus jenerensis	*Stichococcus bacillaris*	CCAP 379/7	AB055864	n/a	-	-	acid springs

Table S2. (continuation)

Clade	Species	Identifier	18S	ITS2	GPS	Land (region)	Habitat
Stichococcus jenerensis	*Stichococcus bacillaris*	D10-1	AB055865	n/a	-	-	-
Stichococcus jenerensis	*Stichococcus jenerensis*	CCAP 379/5	KJ756841	n/a	-	-	marine
Stichococcus jenerensis	*Stichococcus jenerensis*	LU1	KF569726	n/a	-	-	-
Stichococcus jenerensis	*Stichococcus jenerensis*	SAG 2138	DQ275461	this study	-	MY (Kampong Kuala Jenera)	soil (crust on tree base)
Stichococcus jenerensis	*Stichococcus* sp.	FACHB753	EU045358	n/a	-	-	-
Stichococcus jenerensis	*Stichococcus* sp.	HEW2B K3412	this study	n/a	N51°12'36.000" E10°22'11.800"	DE (Hainich-Dün)	soil (forest)
Stichococcus jenerensis	*Stichococcus* sp.	KP09HW3001	this study	n/a	N51°16'17.900" E10°18'38.700"	DE (Hainich-Dün)	subaerial (epixylic; tree bark)
Stichococcus jenerensis	*Stichococcus* sp.	KS126SM6L	this study	this study	S3°58'31.436" W79°4'17.341"	EC (San Francisco)	subaerial (epiphytic; leaves)
Stichococcus jenerensis	*Stichococcus* sp.	MBIC10457	AB183599	n/a	-	-	-

Legend. AQ=Antarctica, AT=Austria, CA=Canada, CH=Switzerland, CN=China, DE=Germany, EC=Ecuador, HR=Croatia, ID=Indonesia, JP=Japan, MX=Mexico, MY=Malaysia, NZ=New Zealand, RU=Russia, US=United States of America.

Table S3. List of the analyzed *Stichococcus*-like clades and their representative strains.

OTU inferred from 18S rDNA similarity ≥ 99.5%; ≥ *99.0%**	Representative strain	Author of denomination	Isolator/year	Synonymic GenBank accessions 18S/ITS2*
OTU8 Diplosphaera*	SAG 48.86	Broady 1983	Broady/1979	*Stichococcus chodati** *Stichococcus chlorelloides* *Chlorella sphaerica**
OTU2 Stichococcus deasonii*	SAG 2139	Neustupa et al. 2007	Deason/1969	*Stichococcus bacillaris* *Diplosphaera* sp.
OTU7 Stichococcus jenerensis**	SAG 2138	Neustupa et al. 2007	Neustupa/2000	*Stichococcus bacillaris*
OTU9 Pseudostichococcus**	SAG 380-1	Moewus 1951	Moewus/1951	*Stichococcus mirabilis** *Stichococcus minutus* *Stichococcus fragilis** *Desmococcus spinocystis** *Diplosphaera* sp.
OTU6 *Stichococcus bacillaris*	SAG 379-1b	Vischer(?)	Vischer/1923	*Stichococcus chloranthus** *Chlorosphaera klebsii* *Gloeotila* cf. *protogenita* *Gloeotila scopulina*
OTU1 *Stichococcus* clade1	KS075SM6T	Hodač 2015	Spitzer/2013	*Stichococcus bacillaris*
OTU2 Stichococcus* clade2	SAG 2482 (=LH08AW8023)	Hodač 2015	Hodač/2008	n/a
OTU3 *Stichococcus* clade3	SAG 2408 (=WB8)	Hodač 2015	Mohr/2005	n/a
OTU4 *Stichococcus* clade4	SAG 2406 (=WB47)	Hodač 2015	Mohr/2005	n/a
OTU5 *Stichococcus* clade5	LH08SW1099	Hodač 2015	Hodač/2008	n/a
OTU2 Stichococcus* clade6	SAG 107.80	Lewin(?)	Lewin/1952	n/a
OTU2 Stichococcus* clade7	LH10HG2063	Hodač 2015	Hodač/2010	n/a

Appendix | Chapter 4

Supporting Figures

Figure S1. ML phylogeny based on 18S rDNA sequences showing the phylogenetic position of *Jenufa* (**Fig. 2**).

Figure S2. ML phylogeny based on 18S rDNA sequences showing the phylogenetic position *Xylochloris* (**Fig. 3**).

Figure S1. Maximum likelihood phylogeny based on 18S rDNA sequences showing the phylogenetic position of *Jenufa* (**Fig. 2**).

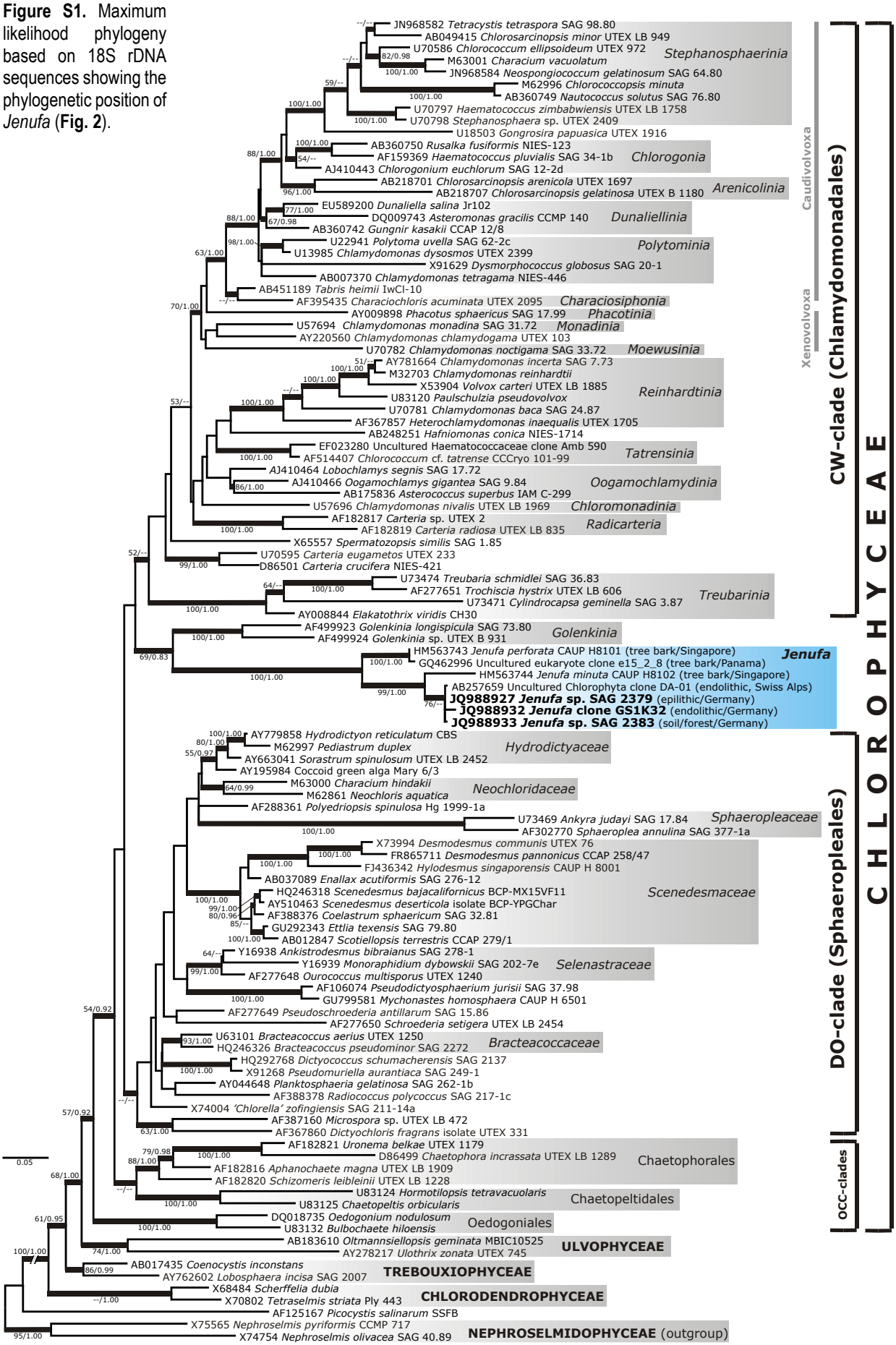
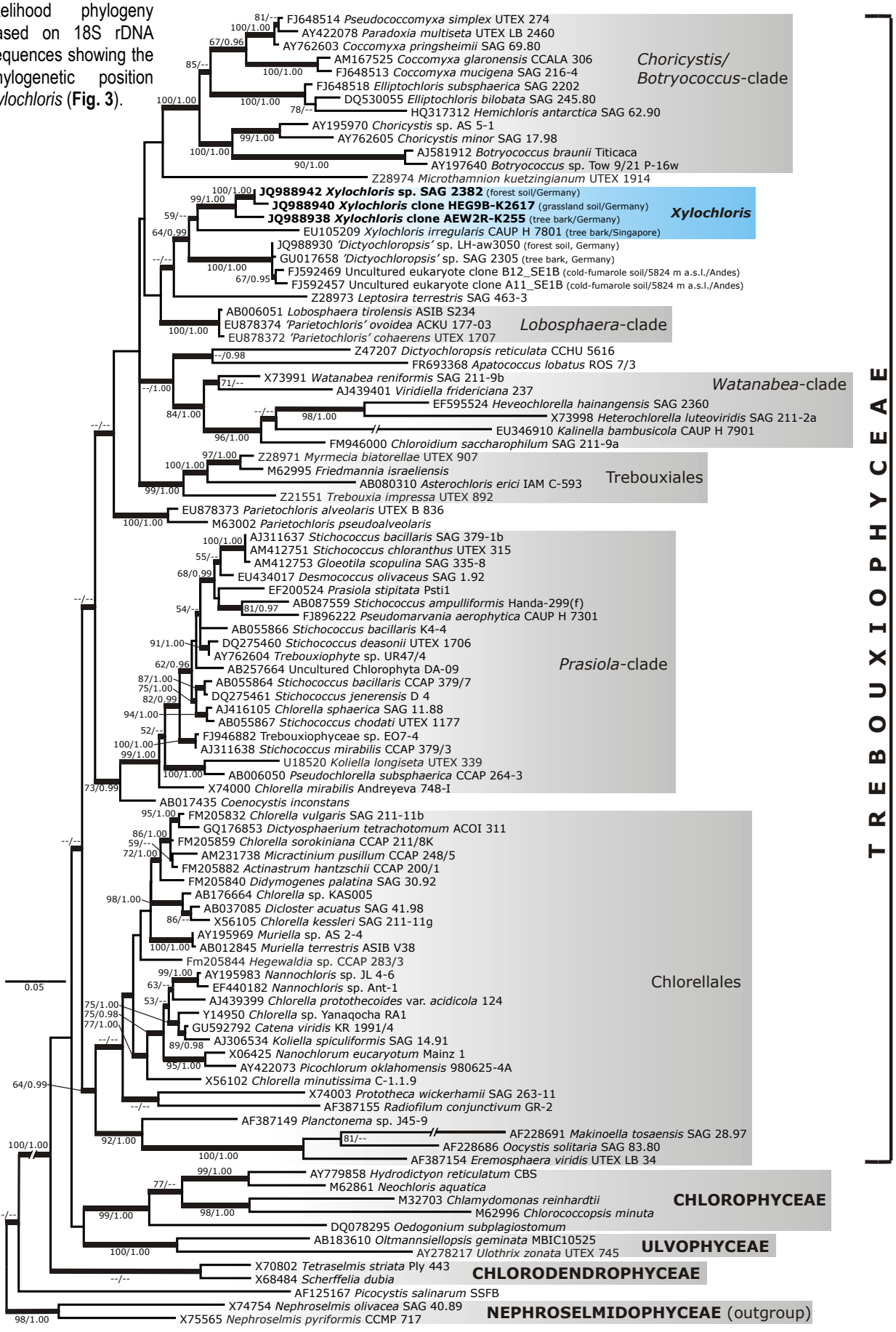


Figure S2. Maximum likelihood phylogeny based on 18S rDNA sequences showing the phylogenetic position *Xylochloris* (**Fig. 3**).



Publications

Contributions to the papers and manuscript drafts included in this thesis

Chapter 1 | Molecular diversity of microscopic Green algae isolated from German soils
(Manuscript draft)
Ladislav Hodač, Christine Hallmann, Karolin Spitzer and Thomas Friedl

University of Göttingen, Experimental Phycology and Culture Collection of Algae (SAG), Göttingen, Germany

Conceived and designed the experiments: LH CH TF
Performed the experiments: LH KS
Analyzed the data: LH, KS
Wrote the paper: LH

Chapter 2 | Diversity of microscopic green algae (Chlorophyta) in calcifying biofilms of two karstic streams in Germany
(Published in Geomicrobiology Journal; doi: 10.1080/01490451.2013.878418)
Ladislav Hodač[1], Nicole Brinkmann[2], Kathrin I. Mohr[3], Gernot Arp[2], Christine Hallmann[1], Jessica Ramm[4], Karolin Spitzer[1], Thomas Friedl[1]

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Conceived and designed the experiments: KM NB TF LH
Performed the experiments: KM NB LH JR KS CH
Analyzed the data: LH KM TF
Wrote the paper: LH TF GA KI NB

Chapter 3 | Phylogenetic analysis of polar *Chlorella* and *Stichococcus* suggests biogeography of airborne microalgae

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Chapter 4 | Molecular evidence for the wide distribution of two lineages of terrestrial green algae (Chlorophyta) from tropics to temperate zone

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